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PB-297 503

Pathogenic 'Naegleria' Distribution in Nature

Epidemiology Research Center, Tampa, FL

Prepared for

Health Effects Research Lab, Cincinnati, OH

May 79

United States
Environmental Protection
Agency

Health Effects Research
Laboratory
Cincinnati OH 45268

EPA-600/1-79-018
May 1979

Research and Development

PB 297503

EPA

Pathogenic *Naegleria*

JUL 11 1979

Distribution in Nature

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TECHNICAL REPORT DATA
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1. REPORT NO. EPA-600/1-79-018	2.
3. RECIPIENT'S ACCESSION NO. PB297503	
4. TITLE AND SUBTITLE: PATHOGENIC NAEGLERIA: DISTRIBUTION IN NATURE	5. REPORT DATE: May 1979 issuing date
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): F.M. WELLINGS, P. T. AMUSO, A.L. LEWIS, M.J. FARMELO, D.J. MOODY, C.L. OSIKOWICZ	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Epidemiology Research Center, 4000 West Buffalo Avenue, Tampa, Florida 33614	10. PROGRAM ELEMENT NO. 1BA607
	11. CONTRACT/GRANT NO. R-804375
12. SPONSORING AGENCY NAME AND ADDRESS: Health Effects Research Laboratory, Office of Research & Development, U.S. Environmental Protection Agency, Cincinnati, OH 45268	13. TYPE OF REPORT AND PERIOD COVERED: Final; Jan. 1975-Dec. 1978
	14. SPONSORING AGENCY CODE: EPA/600/10
15. SUPPLEMENTARY NOTES	

16. ABSTRACT

Infection in man with pathogenic Naegleria, a free-living soil amoeba, results in a usually fatal disease entity known as primary amoebic meningoencephalitis. Epidemiological data usually included exposure to freshwater lakes or streams within the week prior to onset. However, no confirmed isolations had been made from the suspected exposure sites. The major obligation of this study was to determine the presence or absence of pathogenic Naegleria in freshwater lakes in Florida which had been associated with human cases of PAM. Secondary objectives were to elucidate the source of these amoebae, i.e., soil, avian, and/or mammals and to determine the environmental and/or ecological factors related to the presence of pathogenic Naegleria in lake waters.

Results showed conclusively that pathogenic Naegleria amoebae are widely distributed in Florida's freshwater lakes. Examination of samples obtained from freshwater lakes in Georgia also showed extensive distribution of these amoebae. Samples submitted from a lake in South Carolina, which was associated with a PAM case, also yielded pathogenic Naegleria, indicating that these organisms are not unique to Florida's subtropical climate. These studies indicate that pathogenic Naegleria are ubiquitous and over winter in lake bottom sediments or at the sediment/lake water interface. No significant differences have been established among lakes supporting large populations of pathogenic Naegleria and those supporting very limited or undetectable populations.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Amoeba, protozoal diseases, aquatic microbiology, surface waters, lakes, swimming pools	PAM, primary amoebic meningoencephalitis, Naegleria	06F 48G 57H, J, N, U 68D

18. DISTRIBUTION STATEMENT: Release to Public	19. SECURITY CLASS *(This Report)*: unclassified	21. NO. OF PAGES: 91
	20. SECURITY CLASS *(This page)*: unclassified	22. PRICE: PC A05 / MF A01

EPA Form 2220-1 (9-73)

☆ U.S. GOVERNMENT PRINTING OFFICE: 1979-657-060/1667 Region No. 5-11

EPA-600/1-79-018
May 1979

PATHOGENIC *NAEGLERIA*: DISTRIBUTION IN NATURE

by

Flora Mae Wellings
Philip T. Amuso
Arthur L. Lewis
Mary Jane Farmelo
Dewey J. Moody
Caroline L. Osikowicz

Epidemiology Research Center
Tampa, Florida 33614

Grant Nos. R-803511 and R-804375

Project Officer

Walter Jakubowski
Field Studies Division
Health Effects Research Laboratory
Cincinnati, Ohio 45268

This study was conducted
in cooperation with
State of Florida
Central Operations Services
Department of Health and Rehabilitative Services
Tallahassee, Florida 32301

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
CINCINNATI, OHIO 45268

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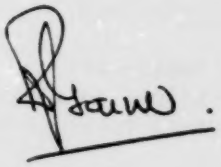
FOREWORD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our national environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The primary mission of the Health Effects Research Laboratory in Cincinnati (HERL) is to provide a sound health effects data base in support of the regulatory activities of the EPA. To this end, HERL conducts a research program to identify, characterize, and quantitate harmful effects of pollutants that may result from exposure to chemical, physical, or biological agents found in the environment. In addition to the valuable health information generated by these activities, new research techniques and methods are being developed that contribute to a better understanding of human biochemical and physiological functions, and how these functions are altered by low-level insults.

This report describes the results of a survey for pathogenic Naegleria amoebae in freshwater lakes, studies on the source of the amoebae, and attempts to determine environmental or ecological factors relevant to the occurrence of the organism in freshwater lakes. A better understanding of these factors may allow development of measures to reduce exposure.



ABSTRACT

Human infection with pathogenic Naegleria, a free-living soil amoeba, results in a usually fatal disease known as primary amoebic meningoencephalitis (PAM). Epidemiological data usually included exposure to freshwater lakes or streams within the week prior to onset. However, no confirmed isolations had been made from the suspected exposure sites. The major objective of this study was to determine the presence or absence of pathogenic Naegleria in Florida's freshwater lakes which had been associated with fatal PAM infections. Secondary objectives were to elucidate the source of these amoebae, i.e., soil, avian, and/or mammals and to determine the environmental and/or ecological factors related to the presence of pathogenic Naegleria in lake waters.

Before field studies could be initiated candidate techniques for concentrating amoeba from water and sediments were modified or devised. Isolation and identification techniques were also evaluated and modified.

Results showed conclusively that pathogenic Naegleria are widely distributed in Florida's freshwater lakes. Examination of samples obtained from freshwater lakes in Georgia also revealed that the distribution of these amoebae was extensive. Samples submitted from a lake in South Carolina, which was associated with a PAM case, also yielded pathogenic Naegleria, proving that these organisms are not unique to Florida's subtropical climate. These studies indicate that pathogenic Naegleria are ubiquitous and overwinter in lake bottom sediments or at the sediment/lake water interface. No significant differences have been established among lakes supporting large populations of pathogenic Naegleria and those supporting very limited or undetectable populations. However, there does appear to be a trend toward increased numbers of pathogens in lakes with increased levels of gram negative bacteria on which pathogenic Naegleria feed.

Laboratory manipulation of the organism has led to a better understanding of the alterations in pathogenicity occasioned by passage of these amoebae on different types of media.

This report was submitted in fulfillment of Grants No. R-803511 and No. R-804375 from the U.S. Environmental Protection Agency and was funded in part by the Department of Health and Rehabilitative Services, State of Florida. This report covers the period 1-1-75 to 12-31-78.

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ACKNOWLEDGMENTS

The authors acknowledge the invaluable contributions made to this study by S. L. Chang, M.D., formerly with the Health Effects Research Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, now retired. His expertise based on years of experience in the field of protozoology was of inestimable value during the initial planning and start-up of this project when staff was being trained and techniques were being developed.

The authors also acknowledge the valuable contributions of Walter Jakubowski, the Project Officer who provided the opportunity to broaden the study to include testing field samples from outside of Florida. His actual participation in the field investigation of the PAM case and his facilitating the lake survey in Georgia are sincerely appreciated. In addition, the overall project has benefited from his critical review of reports and his helpful suggestions.

The studies conducted in Georgia could not have been accomplished without the able assistance of Dr. John E. McCroan, State Epidemiologist and Dr. Maurice Patton, Health Officer, and staff members of the Laboratory and Sanitary Engineering Section of the Georgia Department of Human Resources. Special recognition must be extended to Mr. W. J. Carroll, owner of the recreational site where our first investigation took place, who voluntarily and graciously encouraged the investigation.

In house, Dr. Lillian Stark is recognized for the statistical analyses of data.

SECTION 1

INTRODUCTION

OVERALL OBJECTIVES

Infection in man with pathogenic Naegleria, a free living soil amoeba, results in a disease known as primary amoebic meningoencephalitis (PAM). All but one of the 34 recognized cases which have occurred in the United States have been fatal within six days from the onset of symptoms. Sudden death is always tragic but in the case of PAM, it is compounded by the fact that 32 of the 33 fatalities have involved young, healthy and active individuals between the ages of 18 months and 25 years. One 40-year-old male accounts for the thirty-third fatality.

In the majority of cases, epidemiological evidence indicated that exposure to freshwater lakes or streams had occurred during the week prior to death. Thus, it was assumed that pathogenic Naegleria had been present in the waters to which these individuals were exposed. Irrefutable scientific data to support this hypothesis, i.e., isolation of the organism from exposure sites, had not been published. Therefore, this study was initiated principally to establish a monitoring program to determine whether or not pathogenic Naegleria are present in Florida's lakes. Secondary objectives were to elucidate the source(s) of the amoebae, i.e., soil, avian, or mammals and to determine the environmental or ecological factors related to the presence of pathogenic Naegleria in lake waters.

BACKGROUND DATA

The existence of free living soil amoebae has been recognized for years but their role as a human pathogen was not known until 1965. In 1958, Dr. C. G. Culbertson identified Acanthamoeba in necrotic brain lesions in monkeys and mice.(1) Animals were sacrificed four days after inoculation with amoebae isolated from cell cultures which originally were thought to have contained a virus. In a 1961 publication, Culbertson prophetically suggested that these free living amoebae might cause similar lesions in man.(2)

The first clinical cases of PAM were reported from Australia in 1965.(3) In 1966, Dr. C. G. Butt reported three fatal cases in Florida.(4) Since the causative agent was not isolated from any of these cases, identification was based on stained preparations of brain tissue. The amoebae were identified as Hartmanella strains. In 1968, Butt reported the isolation of amoebae from the brain and spinal fluid of a 16-year-old male who had died in Orlando, Florida.(5) At autopsy, several small fragments of brain tissue and

spinal fluid were inoculated onto agar plates covered with live bacteria. The plates were incubated at room temperature and at 37°C. At the latter incubation temperature, the amoebae grew continuously, whereas at room temperature, only an initial growth phase occurred. Culture methods used for isolating Hartmanella strains, i.e., addition of neomycin or other antibiotics, were unsuccessful, which ruled out the possibility that the amoebae were Hartmanella strains. In addition, the trophozoites could be induced to form flagellates which is a characteristic of the genus Naegleria but not Hartmanella. Because of the foregoing and the mitotic characteristics, the isolated amoebae were considered to be similar to, or possibly identical with Naegleria gruberi, demonstrating for the first time that free living Naegleria were involved in PAM. It has since been ascertained that the Naegleria species was not gruberi but a previously unrecognized species which has been referred to in the literature as Naegleria fowleri,(6) Naegleria aerobia,(7) and Naegleria invadens.(8) Because of the lack of nomenclature consistency, pathogenic Naegleria will be used in this report.

Since that time, many cases have been reported worldwide. In the United States, 34 cases have been recognized. In 1977, three were reported, one each from Georgia, Texas and South Carolina. The Texas and South Carolina cases were the first reported from those states. In 1978, five cases were reported, one in California, two in Florida, one in South Carolina, and one in Pennsylvania. The Pennsylvania case involved a 40-year-old male who had been swimming in Florida and South Carolina lakes before onset of his fatal illness. Only the case reported from California survived following early and extensive treatment. The increased number of reported cases in 1977 and 1978 point to the possibility that because of the national interest in this disease and the improved technologies developed through Federal funding, the disease is more readily recognized and diagnosed.

Based on autopsy and animal data, amoebae enter the brain following intranasal infection. Studies by Chang indicate that pathogenic Naegleria excrete a cytolytic substance which lyses cells, thus providing nutrients for the amoebae.(9) Once infection, i.e., lysis of olfactory mucosal epithelial cells, is initiated, the amoebae pass directly along the olfactory nerve plexus to the brain.

Before this study began, no data were available on the distribution of pathogenic Naegleria in the environment. In 1975, de Jonckheere, et al., reported that a single pathogenic Naegleria was isolated from a thermally polluted canal in Belgium(10) which was considered to have been the exposure site for a boy who died of PAM in 1973.(11) During their studies, another Naegleria species was isolated which was shown by the indirect fluorescent antibody (IFA) technique(12) to be antigenically closely related to the pathogenic Naegleria. However, this new isolate did not produce fatal infections in mice when instilled intranasally.

STUDY RATIONALE AND APPROACH

Florida with its semitropical climate and innumerable freshwater lakes, both natural and man made, offered a year round study site for this project. The State of Virginia had reported more cases of PAM(13) than had Florida.

However, most of the cases in Virginia appeared in epidemic form as opposed to the endemicity of Florida's cases. Thus it appeared that an ecological study in Florida should offer a relatively good possibility of success.

Initially, techniques had to be modified or developed for concentrating amoebae from large volumes (189 to 278 liters) of water and from lake bottom sediment samples. Isolation media and incubation temperatures were tested to determine optimal conditions and immune sera prepared for identification procedures including IFA.

Once reliable techniques were developed for isolating amoebae from throat and stool swabs, early studies were directed towards elucidating the role of avians and mammals in the dissemination of the organism. Extensive sampling was undertaken to determine the distribution of pathogenic Naegleria in Florida's lakes. Various chemical and physical parameters were investigated in an attempt to characterize those lakes yielding pathogenic Naegleria.

SECTION 2

CONCLUSIONS

These studies have shown conclusively that pathogenic Naegleria amoebae are widely distributed in freshwater lakes. Over 50% of the water tested from lakes in which the temperature was 30°C or greater, yielded the amoeba from one or more water or bottom sediment samples. This amoeba species has the propensity to proliferate in warm waters. Therefore, thermal enrichment, i.e., increasing natural water temperatures by the addition of cooling waters, has been implicated in a fatal case of PAM in Belgium.(10) The role of thermal enrichment in the proliferation and maintenance of pathogenic Naegleria in Florida's lakes is unimportant because natural lake water temperatures have at times surpassed those recorded in the thermally enriched lakes studied. The number of isolates obtained from measured quantities of water from thermally enriched and natural waters showed larger populations in the latter. Laboratory studies demonstrated that variations in temperature were more deleterious to the cysts than were either constant high or low temperatures. Under the latter condition, encystation occurred, thus, insuring survival over time. Since the temperature of the thermally enriched waters in this study tended to vacillate extensively, this may explain the lower pathogenic Naegleria population levels demonstrated.

No evidence could be found for implicating avians or mammals in the dissemination of this organism in nature. No amoebae could be recovered from stools of various avians and mammals inoculated with large numbers of pathogenic Naegleria. This does not rule out the possibility that these and other avian or mammalian species not tested may play a role in dissemination, perhaps mechanical if not biological. However, the wide distribution of the organism as seen in the number of lakes yielding the amoebae in a single sampling would appear to indicate that these pathogenic amoebae are ubiquitous in Florida. Isolates obtained from lake bottom samples during winter months demonstrated the mechanism for over-wintering, i.e., encystment and residence at the lake bottom/water interface or in the upper few centimeters of the lake bottom sediments.

Two distinct antigenically related Naegleria species have been confirmed. IFA testing showed extensive antigen sharing but specific antigens were demonstrable using heterologous absorbed serum. Growth studies showed distinct differences in motility, plaque size, and colony populations. Sonic disruption of cysts of the nonpathogens required approximately 50% greater shearing forces than did cysts from the pathogenic Naegleria strains. Differentiation of the seropositive nonpathogenic and pathogenic strains is readily accomplished. The latter produce luxuriant growth in Chang's calf serum-yeast-

extract-casein medium (CSYE:M)(8) in 24 to 48 hours (see Appendix B), whereas minimal to no growth results with the former. In addition, intranasal instillation of the seropositive nonpathogenic Naegleria does not produce a lethal infection in mice.

Chemical analyses of various lake waters have failed to elicit significant differences among lakes supporting as opposed to those not supporting pathogenic Naegleria growth. There are preliminary indications that the concentration of gram negative organisms may be related to population levels of pathogenic Naegleria.

The relatively high numbers of pathogens present in several of the lakes tested, i.e., one pathogenic Naegleria per 25 ml of water tested and 6 per 25 ml in one of the lakes, would appear to pose a real threat to individuals swimming in such lakes. However, no cases of PAM have been associated with these lakes since this study was initiated in spite of the fact that many individuals were observed swimming in these lakes at the time samples were obtained.

Samples of water or bottom sediments from lakes associated with fatal cases reported from several states since July, 1977, were processed for amoeba isolation. Pathogenic Naegleria were recovered from all exposure sites with the exception of those in Texas and California. In the latter instances, samples were in transit for longer periods which may have encouraged overgrowth of other amoebic and/or bacterial species.

SECTION 3

RECOMMENDATIONS

There is now ample evidence that pathogenic Naegleria are widely distributed in lakes in Florida and Georgia and probably throughout the southern United States. Increased ambient temperatures resulting in increased lake water temperatures enhance the proliferation of pathogenic Naegleria. Why this is true in some lakes and not in others should be ascertained. A thorough evaluation should be made of lakes in which pathogenic Naegleria proliferate and those in which few, if any, are found. This should include limnological studies as well as enumerations of gram negative organisms on which pathogenic Naegleria feed. A determination of all amoeba species and ciliates present in the study lakes should be made in an attempt to identify predators.

Lakes should also be carefully evaluated for pollution sources both direct and indirect. Preliminary evidence that increased populations of gram negative organisms enhance pathogenic Naegleria proliferation appears to indicate that pollution plays a role in population densities. If this can be shown conclusively, control measures could then be devised and instituted.

Relatively little data are available on the seropositive nonpathogenic and pathogenic Naegleria amoebae per se. Nutritional and enzymatic studies of both species should be undertaken to identify the biochemical differences between these organisms. The possibility of an endosymbiont in the pathogenic Naegleria which permits bypassing the growth factor(s) required by the seropositive nonpathogen should be investigated. More refined studies of cyst survival under anaerobic conditions should be done to conclusively determine whether cysts are facultative or just tolerant under anaerobic conditions.

Modes of dissemination of pathogenic Naegleria in nature should be studied further. Various species of mammals and avians not yet tested and perhaps, reptiles may play a role in introducing either by mechanical or biological means, pathogenic Naegleria into lake waters.

Differences in pathogenicity of isolates from surface waters versus water nearer the bottom should be studied, particularly in lakes where infections had occurred. Quantitation of organisms at the two levels should also be determined. Epidemiological evidence continues to accrue indicating that infection occurs in those people who swim near the bottom of lakes. Whether this is related to the number of organisms to which the swimmer is exposed or to increased pathogenicity of the organism is not known. Only

when sufficient data are available to permit a thorough understanding of the organism *per se* and the chain of events leading to infection in man can control measures be instituted.

SECTION 4

MATERIALS AND METHODS

At the initiation of this study, limited data were available on field techniques applicable to the concentration and isolation of pathogenic Naegleria from field specimens, particularly from lake bottom sediments and large volume water samples. Thus, much time and effort were devoted to devising or adapting and testing techniques before the field work was started.

Data presented in this section include the various approaches used in the development of techniques and the rationale for them. Studies were not necessarily done in the order of presentation but rather are grouped on the basis of relatedness.

SAMPLING SITES

Sampling sites were selected initially on two bases (1) thermal enrichment, and (2) suspected exposure sites for fatal cases of PAM. One thermally enriched site which received electric plant cooling waters and two natural lakes which had been considered to be the exposure sites for three fatal cases of PAM were sampled initially. As the study progressed a larger number of lakes were tested. Samples of lake water and bottom sediments were obtained from both shallow and deep water areas. Depending on the extent of sampling at each lake and the geographical distances between sites, one or several lakes were sampled on a single day. Quantitative data were based on multiple, measured samples and on the most probable number technique which was adapted to this study.

STOCK CULTURES

Stock cultures of McM, MO (Australian isolates), HB_1, RL and GJ (Florida isolates) were received from S. L. Chang, M.D., Environmental Protection Agency, Cincinnati, Ohio. All strains had originally been isolated from fatal cases of PAM. Cultures were received and maintained on inorganic agar seeded with Enterobacter aerogenes. Routine passages were made as necessary for maintaining the strains. Strain 43-9 used in these studies is a seropositive nonpathogenic Naegleria strain isolated in February, 1976, from a 100 ml sediment sample obtained from a thermally enriched lake.

CULTIVATION TECHNIQUES

To determine optimal conditions for isolation of pathogenic Naegleria from field specimens, various cultivation parameters were investigated as

described below.

Incubation Temperature

Temperatures from 35°C to 46°C were chosen as possible incubation temperatures. Inoculation of four stock cultures containing both trophozoites and cysts into CSYECM for axenic cultivation of pathogenic Naegleria resulted in little or no propagation of the organism at 46°C. However, satisfactory growth occurred at 35°C. Cysts of four stock cultures induced by holding trophozoite cultures at 4°C for five days were inoculated into CSYECM and incubated at 35°C, 42°C, and 44°C. Excystation and trophozoite replication occurred at all temperatures, but the amount of growth was inversely proportional to the temperature of incubation, few trophozoites being noted at 44°C and most prolific growth occurring at 35°C.

Two final temperature-growth experiments utilizing five stock cultures of trophozoites and cysts and four stock cultures of cysts only, showed excellent growth at 35°C, good growth at 43 to 44°C, and poor growth at 46°C. From these studies it was concluded that incubation of field samples should be carried out at 43 to 44°C to favor propagation of Naegleria and to discourage growth of undesirable saprophytes.

Antimycotic Agents

Large numbers of saprophytes and other undesirable microflora that naturally contaminated field specimens overgrew pathogenic Naegleria at 37°C. Various antimycotic agents were tested to determine their inhibitory effect on these interfering species.

Various dilutions of Nystatin, Actidione and 5-Fluorocytosine were incorporated into CSYECM. Both Nystatin and Actidione inhibited pathogenic Naegleria growth at extremely low concentrations and were therefore eliminated. However, concentrations of 5-Fluorocytosine as high as 1,400 µg per ml permitted good growth of Naegleria. When a saprophytic fungus isolated from a throat swab taken from a bird was introduced into CSYECM containing 1,400 µg of 5-Fluorocytosine and incubated at 37°C, saprophytic growth was not inhibited. Because laboratory strains of Naegleria showed good growth at 43 to 44°C and the growth of temperature sensitive saprophytic microflora was somewhat inhibited, antimycotic agents were not incorporated into the medium.

Antibiotic Concentration in CSYECM

The effect of various levels of antibiotics on amoebic growth was determined in anticipation of the use of CSYECM for isolating amoebae from nasal exudates of swimmers exposed to pathogenic Naegleria contaminated water. Table 1 shows results of growth studies in which varying doses of penicillin and streptomycin were added to CSYECM. Even with the highest levels of antibiotics used, no toxic effects were noted. Therefore, with exceedingly contaminated specimens, large doses of antibiotics were used with impunity.

TABLE 1. ANTIBIOTIC EFFECT ON GROWTH OF PATHOGENIC NAEGLERIA

Pathogenic strain	Total inoculum	Amoeba concentration at 48 hours in media containing the following U/ml of penicillin and µg/ml of streptomycin			
		100	300	400	500
GJ	2.9×10^4	4.6×10^5	5.1×10^5	5.1×10^5	3.7×10^5
83-16*	5.8×10^4	1.7×10^6	1.8×10^6	1.6×10^6	1.7×10^6
77N-475**	4.4×10^4	8.5×10^5	4.9×10^5	6.6×10^5	6.2×10^5

* Pathogenic Naegleria isolated from field specimen February 24, 1976.
** Pathogenic Naegleria isolated from field specimen June 28, 1977.

Growth Media

Sensitivities of CSYECM and inorganic agar plates spread with a lawn of living E. aerogenes were investigated. Varying two-fold dilutions of an HB_1 trophozoite suspension were titrated on both media. Only one tube containing CSYECM and one inorganic agar plate were used for dilutions 10^{-1} through $10^{-3.6}$ but four replicate tubes and plates were used for dilutions $10^{-3.9}$ through $10^{-6.6}$. The 50% end points for the two media were comparable, $10^{-5.5}/0.2$ ml for CSYECM and $10^{-5.4}/0.2$ ml for the inorganic agar. Since sensitivities were relatively equal, inorganic agar with E. aerogenes was selected for use with field specimens.

CONCENTRATION TECHNIQUES

Throat and Rectal Swabs or Feces

Before the role of avians or mammals in pathogenic Naegleria amplification or dissemination in nature could be evaluated, techniques for concentrating the organism from throat and rectal swabs or feces had to be developed.

Normal duck feces were inoculated with 10^6 Naegleria. Three simulated rectal swabs were prepared from the inoculated feces and processed individually as follows. A swab was placed in 10 ml of sterile antibiotic water (SAW) containing 200 U penicillin, 200 µg streptomycin, and 50 µg aureomycin ml^{-1}. After vigorous agitation the suspension was serially filtered through 45 µm, 10 µm, and 8 µm porosity membranes at a centrifugal force of 1,200 X g. The latter two membranes were washed by passing 10 ml of SAW through the membrane in situ using the above relative centrifugal force.

Subsequently, individual membranes were placed in 5 ml aliquots of SAW and shaken to suspend any membrane-attached organisms. Both the suspending SAW and membrane (8 µm and 10 µm) were inoculated separately into CSYECM. Organisms were recovered from both membranes and the suspending medium,

demonstrating incomplete elution from the membrane. An enterococci appeared on day two post-inoculation and subsequently interfered with replication of the recovered Naegleria.

Similar experiments using centrifugal force filters were conducted; in these, the organisms were recovered from the suspending SAW in pure culture but membranes yielded enterococcus contaminated Naegleria cultures. This contamination was overcome by increasing ten-fold the concentration of penicillin and streptomycin in the SAW, by increasing the time of membrane agitation on a rotary shaker to one hour at room temperature, and by incubating this suspension for an hour at 37°C prior to inoculation into CSYECM.

To determine the sensitivity of this isolation technique, two serial ten-fold dilutions of trophozoites were made in distilled water and concentrated. Back titration of the original dilutions showed 10^5 and 10^4 organisms per ml. After centrifugal concentration on 8 µm porosity membranes, the yield was 10^4 and $<10^3$, respectively, from the two inocula. The question of trophozoite loss into the filtrate due to G forces was addressed in subsequent experiments by examination of the filtrate. No organisms were detected, and results of these latter experiments also showed a 10% recovery rate. Although the reduced recovery yield has not been explained, it may be due to several factors, i.e., trophozoite death due to manipulation, adherence to glassware, etc.

Lake Waters

Initially, 3.78 liters (one gallon) of lake water were passed through a series of nylon mesh cloth screens having pore sizes of 45 µm and 10 µm. After each filtration, the screens were flushed with sterile distilled water and the filtrate homogenized in a Waring blender. Organisms present in the final filtrate were concentrated by centrifugation. This technique (Dr. S. L. Chang, personal communication) proved to be time consuming and severely limited the sample size. To facilitate large volume sampling, a sand column technique was developed.

A column 40 cm in length and 5 cm in diameter containing 600 ml of Ottawa silica sand was prepared. Cysts and trophozoites of the McM strain of pathogenic Naegleria were inoculated into a gallon of distilled water and passed through the column at a pressure of 5 psi. Sand from the column was removed and deposited in a beaker into which 700 ml of 1% beef extract was added. After the mixture was stirred vigorously for ca 15 minutes, the sand was permitted to settle, and the supernate was decanted into centrifuge bottles. After a series of centrifugations of the supernate, the final pellet was resuspended in 1 ml of distilled water. Back titration of the inoculum in CSYECM indicated that $10^{5.3}$ viable organisms had been present in the original inoculum, but that only 10% of these ($10^{4.3}$ amoebae) were recovered from the sand column.

To determine distribution within the column, a gallon of Lake Conway water inoculated with $10^{7.3}$ trophozoites was forced through a sand column which was encased in tygon tubing (15.9 mm internal diameter by 598 mm). The tubing was sliced at one-inch intervals and the sand contained in each

section assayed separately. Organisms were detected in all samples, but percent of recovery was not established.

Because of the extensive distribution of organisms throughout the column, amoeba losses would be expected during filtration. After it was passed through the column, the first liter of water from a lake known to contain pathogenic Naegleria was collected. A second liter was obtained after 378.5 liters had been filtered, and a third, after 1,135.6 liters. Only the last liter collected yielded pathogenic Naegleria. Based on these data, field samples were limited to a maximum of 189.2 liters to minimize loss of amoebae from the column.

The refined technique used during the field studies has been described in detail elsewhere.(14) A polyvinylchloride (PVC) pipe 38.1 mm in diameter and 60 cm long was filled with approximately 600 ml of Ottawa silica sand to within 10 cm of the top. With a small, high volume pump (Myers Mod. #M7 powered by a 3 H.P. gasoline engine, Briggs and Stratton Mod. #80200) on site, 189.2 liters of lake water were forced through the sand column at approximately 3.78 liters/min. A 45 µm mesh cloth placed over the bottom of the column and secured with a hose clamp prevented loss of sand through the bottom.

Small Water Samples

One to two liter water samples were obtained in sterile glass jars and returned to the laboratory for processing within four hours. These were filtered through individual 5 µm porosity membrane filters (Millipore Corp., Bedford, Mass.). The filter was inverted onto an inorganic agar plate (IA) seeded with E. aerogenes.

Sediment Samples

Lake bottom sediments were obtained in one of three ways(14) depending on the depth of the water. In shallow areas, a small can was used to scoop up the desired sample. In depths from 1.2 to 2.4 m, cores were obtained with a PVC pipe. In deep water, drag samples were obtained using a weighted can.

Sand from the columns and sediment samples were placed in individual resealable plastic bags for transport to the laboratory. Initially, samples were processed immediately. As the study progressed, samples were held at 43°C from 18 to 96 hours before processing.

ISOLATIONS

These techniques have been reported in detail elsewhere(14) but briefly, each of the mud and sand column specimens were vigorously stirred in 1% beef extract. Following settling of the sand and sediments, the supernates were decanted into centrifuge tubes and centrifuged at 600 x g for 20 minutes. The supernate was then discarded and the sediments inoculated onto sufficient IA plates covered with live E. aerogenes to permit inoculation of all sediments, approximately 20 plates per sample. These were incubated in closed petri dish cans at 43 to 44°C.

IDENTIFICATION

These procedures have been described in detail elsewhere.(14) Plaques were examined for morphology, and those showing a sharp, even border were tentatively considered to be pathogenic Naegleria if the trophozoites showed typical limax form and eruptive-like pseudopods. Trophozoites were tested for flagellate transformation. Cysts were carefully examined to determine the presence of pores. Final identification was based on the trophozoite growth pattern in CSYECM, on their antigens as shown in the indirect immunofluorescent antibody (IFA) test, and on their pathogenicity for mice following intranasal instillation.

MOUSE INOCULATION

All isolates identified as pathogenic Naegleria based on IFA and growth in CSYECM were not tested for pathogenicity in mice because of the large number of isolates obtained. Usually, only one isolate from each positive field sample was confirmed by mouse inoculation. Details of the actual procedure have been described elsewhere.(14) In all instances, when intranasal instillation of trophozoites resulted in mouse fatality, brains were harvested and the cause of death confirmed by microscopic visualization of the amoebae or reisolation on IA.

INDIRECT FLUORESCENT ANTIBODY TEST

The technique used was a modification of that described by Van Dijck, et al.(12) Serial dilutions of hyperimmune rabbit antiserum prepared against the GJ strain were reacted with acetone fixed trophozoites on a slide. Fluorescein-labeled anti-rabbit globulin was then added. Following the prescribed reaction period, slides were washed, air-dried, and coverslips were mounted. Reactions were read through a microscope equipped with an HBO-200 light source, a dark field condenser and filters (BC-38, OG1 and a Wrattin 2B eyepiece filter). Normal rabbit serum was used as a control. The GJ antiserum absorbed with 43-9 trophozoites was included in certain of the tests.(14)

SECTION 5

RESULTS AND DISCUSSION

This study represented the first large scale field investigation initiated in the United States to determine the natural distribution of pathogenic Naegleria. Because no background data were available, techniques were developed or adapted and evaluated. Once techniques were established, infection experiments were carried out and rectal and throat swabs obtained from wild-caught mammals were examined. This was followed by a full-scale study of thermally enriched lakes and four natural lakes which had been considered as exposure sites for PAM cases. Such lakes are referred to as suspect lakes in this report. When it was recognized that pathogenic Naegleria might be more widespread than originally believed, a survey of freshwater lakes in Florida was undertaken.

INFECTION EXPERIMENTS

An Indian Runner duck, Muscovy duck, a raccoon, and an opossum were inoculated with pathogenic Naegleria, HB_1 strain. The inoculum was deposited into the crops of the ducks, but was instilled intranasally in the mammals. All daily rectal and nasopharyngeal swabs were negative for the agent. The animals' drinking water was also tested and only one specimen, obtained from the raccoon's cage two days after inoculation, was positive for pathogenic Naegleria. No immune response was evidenced in the microscopic agglutination test(12) of sera from any of the inoculated subjects and no isolations of pathogenic Naegleria were made from necropsy tissues tested.

ANIMAL ISOLATION ATTEMPTS

Rectal and throat swabs from wild-caught raccoons and opossums chosen because of their propensity for visiting freshwater shores and shallows were tested for pathogenic Naegleria. In all, 137 throat and rectal swabs were collected and processed. Ten yielded amoebae when inoculated onto IA plates. However, only one plaque obtained from the culture of a pharangeal swab taken from a raccoon gave rise to amoebae which could be induced into flagellate transformation. These amoebae were identified as Naegleria gruberi. None of the isolates could be propagated in CSYECM and none resulted in fatality following mouse intranasal instillation. A large amoeba (probably Acanthamoeba) from each of two raccoons (rectal swabs) was pathogenic for 3 to 4 week old white Swiss mice by the intracerebral route. Although the concentration technique was only 10% efficient, the total absence of pathogenic Naegleria in the 137 specimens tested would appear to indicate that the species tested do not play a major role in amplification of pathogenic Naegleria in nature.

However, these species could possibly play a role in dissemination of the amoebae by mechanical transfer from lake to lake.

ISOLATION FROM NATURAL AND THERMALLY ENRICHED LAKES

The results for all Florida lake samples processed are shown in Tables A-1 through A-8 (Appendix A). These data show conclusively that pathogenic Naegleria are widespread throughout Florida. Each of the four lakes suspected of having been the exposure site for PAM cases supported large populations of pathogenic Naegleria. One of the two thermally enriched lakes which was sampled frequently yielded the agent but the other, which was sampled only once, was negative. Of the ten lakes which were sampled in an attempt to locate a negative control lake, only Lakes Corner, Jessup and East Tohopekaliga did not yield pathogenic Naegleria. Of these three only Lake Corner was sampled frequently and at depths which should have been positive had the lake been heavily populated. Even so, no samples were obtained during the summer months which would be necessary before the lake could be considered as negative. Lake Beauclaire, one of the seven positive lakes in this group, yielded a single isolate from a sediment sample taken at a water depth of ca 1.2 m. Summer sampling of this lake would also be required if this lake were to be considered as a control lake having a low population of pathogenic Naegleria.

Lakes that were surveyed, i.e., sampled only once or twice to determine the geographical distribution of the agent, were categorized based on their location in or outside of Orange County. These lakes were all sampled at least once between June and October. Lakes in northern Florida were sampled in June and August because of the lower ambient temperatures that occur in September and October in that section. Even under these conditions, only 40.4% (21/52) positivity was shown in lakes outside of Orange County, whereas, 81.2% (13/16) of lakes in Orange County yielded pathogenic Naegleria (see Table 2).

TABLE 2. NUMBER AND PERCENT OF LAKES YIELDING PATHOGENIC NAEGLERIA IN THE FLORIDA SURVEY

		Number of lakes positive				
Surveyed lakes	Number tested	Water only	Sediment only	Water and Sediment	Total	Percent
Outside Orange County	52	10	5	6	21	40.4
In Orange County	16	8	0	5	13	81.2

Sample size and water temperature may have influenced these results. Because all sediment samples in this survey were from shallow water sites, the variability due to depth was held constant.

The relationship of water temperature and sample size to positivity in surveyed lakes is shown in Tables 3 and 4. Four 0.25 ml water samples from Orange County lakes and one 100 ml sediment sample from a lake outside of Orange County all obtained in February were excluded from these tables. Large water samples (189.2 L) from the lakes surveyed outside of Orange County (see Table 3) yielded positive results in 46.1% (6/13) of the tests, whereas one and two liter samples were positive in 12.5% (4/32) and 15.4% (4/26) of the tests respectively. Average and median water temperatures were lower in lakes from which large samples were obtained, yet a higher percentage of positivity was shown. Therefore, a larger number of lakes might have been positive had large samples been obtained from lakes surveyed outside of Orange County. The fact that data from Orange County lakes surveyed did not show this difference would appear to indicate that populations of pathogenic Naegleria were greater in Orange County than in other Florida lakes. In addition, results obtained from two liter samples also appear to indicate the presence of larger populations of this amoeba in Orange County lakes. In spite of the fact that the water temperatures averaged over 30°C in lakes outside of Orange County only 4 of 26 two liter samples were positive. Whereas, in Orange County lakes where the water temperature averaged only 26.7°C, both two liter samples were positive. If these indications of increased population levels were to be confirmed, it could help to explain why five of the nine PAM cases occurred in Orange County.

TABLE 3. RELATIONSHIP OF WATER TEMPERATURE AND SAMPLE SIZE TO POSITIVITY SHOWN IN THE LAKE SURVEY

Sample size (liters)	# Positive/ # tested	Percent positive	Average temperature		Median temperature	
			Positive samples	Negative samples	Positive samples	Negative samples
Lakes outside of Orlando						
1	4/32	12.5	31.9	32.0	32.0	32.2
2	4/26	15.4	30.1	30.7	30.7	31.0
189.2	6/13	46.1	29.0	29.4	28.5	29.0
Orlando lakes						
2.0	2/2	100	26.7	--	--	--
94.6	4/4	100	31.1	--	31.0	--
189.2	8/14	57.1	29.8	27.8	29.5	29.0

The major variables affecting the sediment sample results were size and water temperature because, as previously stated, all samples were obtained from comparable, shallow water sites. In contrast to the large water samples which appeared to offer a better opportunity for isolating pathogenic

Naegleria, large sediment samples appeared to offer the least possibility. None of ten 600 ml samples obtained in June were positive, whereas 10% of the 300 ml samples obtained during the same time period yielded pathogenic Naegleria. In August, 33.3% of the 100 ml samples obtained from lakes outside and 75% of those from lakes in Orange County were positive, whereas none of ten 400 ml samples tested during August yielded the agent. These findings suggest that elution of the amoeba from large samples may be less efficient than from the small ones or, amoebae which had been eluted may be trapped as the larger quantities of sand settle out. The validity of this observation requires more refined experimentation.

TABLE 4. RELATIONSHIP OF SAMPLE SIZE AND WATER TEMPERATURE TO POSITIVITY OF SEDIMENT SAMPLES IN LAKES SURVEYED

Month	Average water temperature (°C)	Sample size (ml) 100 Percent positive	200 Percent positive	300 Percent positive	400 Percent positive	600 Percent positive
Lakes outside of Orlando						
June	31.4	--*	--	10.0 (2/20)**	--	0.0 (0/10)
July	N.R.†	--	--	0.0 (1/1)††	--	--
Aug.	30.9	33.3 (3/9)	7.1 (1/14)	--	0.0 (0/10)	--
Sept.	29.6	75.0 (3/4)	14.3 (1/7)	--	--	--
Oct.	26.8	0.0 (0/4)	25.0 (1/4)	--	--	--
Lakes in Orlando						
Aug.	31.1	75.0 (3/4)	--	--	--	--
Sept.	29.9	--	27.3 (3/11)	--	--	--
Oct.	26.6	0.0 (0/2)	--	--	--	--

* No samples.
** Number positive over number tested.
† Not recorded
†† Companion 3.2 liter water sample was positive.

In examining the percent of positive water and sediment samples as related to temperature from all natural lakes (see Figure 1), there is ample evidence of a direct correlation between positive water samples and elevated water temperatures. Positive sediment samples showed less correlation with temperature, having a slightly higher positivity rate in February and March when the average water temperatures were 15.3°C and 21°C respectively, than in September when the average water temperature was 30.5°C.

Positive sediment samples showed a relatively good correlation with depth. Figure 2 shows this relationship for sediment samples from all lakes tested excluding the two thermally enriched lakes. It should be noted that in depths of 6.1 m to 9 m, over 50% of all samples taken yielded pathogenic

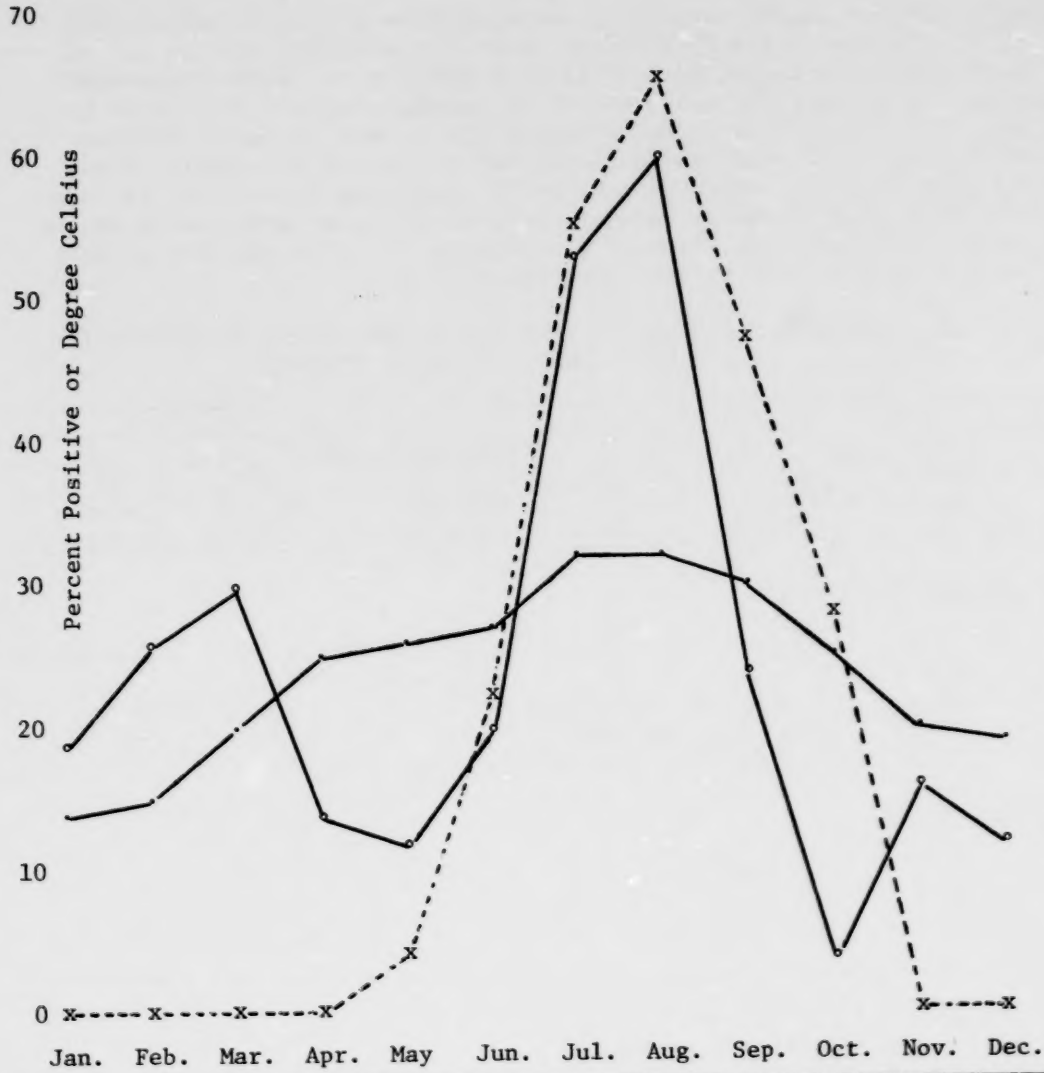

Figure 1. Percent of positive water and sediment samples as related to the average water temperature by month. (·—·) average water temperature; (x--x) percent of water samples positive; (∘——∘) percent of sediment samples positive.

Naegleria. All positive samples from the 0 m to 1 m depth were obtained from June through October (see Table A-9, Appendix A). Conversely, positive samples from all other depths were obtained from October through June except for the 13 samples obtained from the 2.1 m to 3 m depths in July, all of which were positive. This would appear to indicate that had more sediment samples been taken during the warmer months, the overall percentage of positivity would have increased, particularly for the shallow areas (0 m to 3 m). These findings accentuate the importance of testing sediment samples from deep

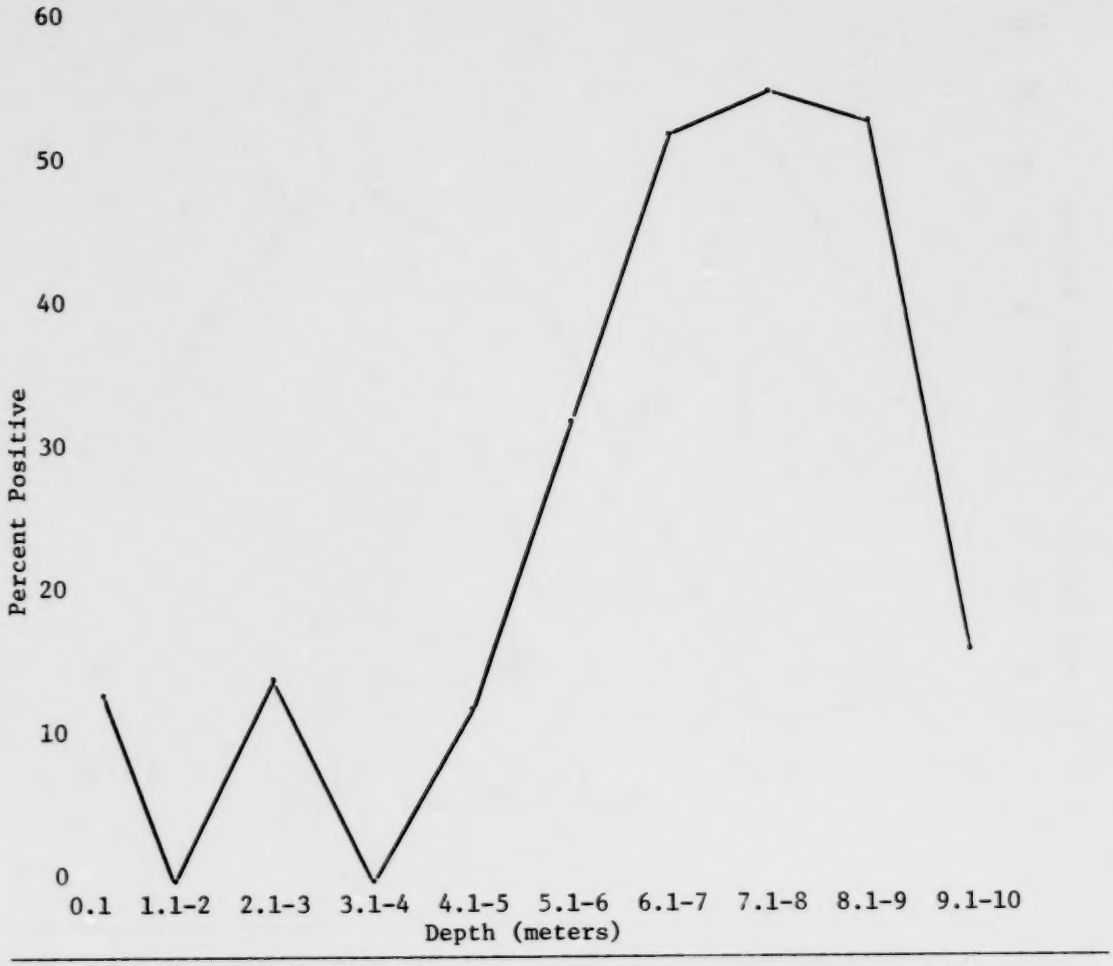

Figure 2. Percent positive of sediment samples from all natural lakes compared with water depths.

water sites during the cooler months.

In Lake Conway, over 40% of all sediment samples obtained from water depths of 4 m to 10 m were positive (Figure 3). A comparison of the dotted line representing the actual number of samples tested with the solid line representing the percent of samples which were positive rules out bias in the sampling procedures; that is, of 96 samples obtained from water depths of 6.1 m to 8 m, 81.2% (78/96) were positive, whereas of 127 samples obtained from water depths of 0 m to 4 m, only 6.3% (8/127) were positive. These data from a single lake confirm the hypothesis that overwintering of the pathogenic Naegleria does occur in deep water sediments or at the sediment/water interface. The inability to demonstrate pathogenic Naegleria in the water column or in sediments obtained from shallow water sites during cool weather

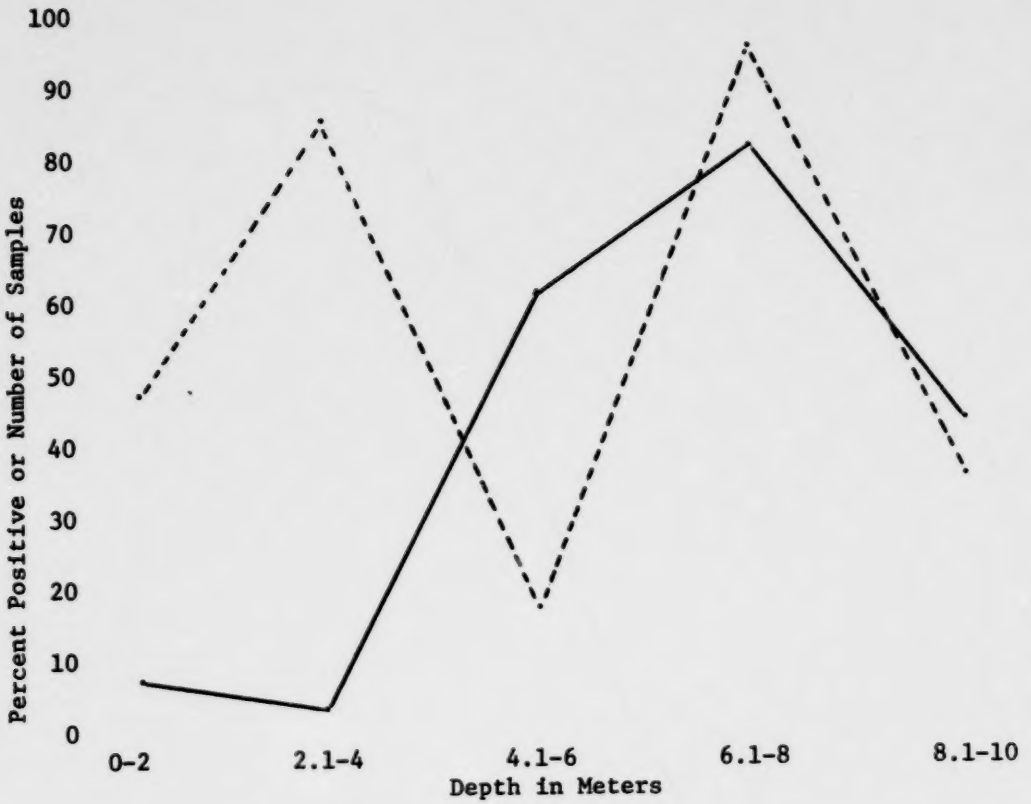

Figure 3. Number of sediment samples tested and percent positive from Lake Conway - January, 1976 - March, 1978. (----) number of samples tested; (——) percent positive.

is no indication that the amoebae have disappeared from the lake.

Some investigators have incriminated thermal enrichment in the maintenance and proliferation of pathogenic Naegleria in nature. However, our data do not support this hypothesis in Florida. Laboratory studies have shown that fluctuating temperatures are detrimental to pathogenic Naegleria cysts. Four comparably heavy plate cultures of the pathogenic GJ strain were prepared and held at 35°C. This resulted in encystment of the trophozoites by day four. Cysts in a circumscribed, marked area on each plate were counted and then each plate was subsequently exposed to a different temperature, i.e., one plate was held at a constant 35°C, one at 22°C, and another at 4°C. The fourth plate was subjected to alternating temperatures of 22°C for 8 hours, then 4°C for 16 hours. Cysts within the marked areas were observed daily and viable cysts counted. Refractile cysts were considered viable. This

criterion was verified by random testing of refractile versus non-refractile cysts through inoculation onto IA plates seeded with E. aerogenes. As shown in Table 5, only the 35°C temperature was more detrimental to the cysts than the alternating temperatures, 7.7% and 16.7% survival respectively. At 22°C, 51.2% of the cysts remained viable over the 35 day period while at 4°C, 81.3% survived. These experiments should be repeated using other strains, particularly recently isolated ones. Strain differences may alter the data but because of the gross differences noted, it is doubtful that the deleterious effect of temperature fluctuation would be negated by strain differences. These data as they stand, suggest that pathogenic Naegleria population levels would be lower in thermally enriched lakes than in natural lakes because of temperature fluctuations in the former. Previously published data showed that the thermally enriched lake water temperatures fluctuated rapidly and extensively, depending on the frequency of thermal water discharge. In natural lakes, a more uniform water temperature curve rather closely reflected ambient temperatures.(14)

TABLE 5. TEMPERATURE EFFECT ON PATHOGENIC NAEGLERIA CYST SURVIVAL

No. of days exposure	Percent of viable cysts after daily exposure at			
	35°C	22°C for 8 hrs. 4°C for 16 hrs.	22°C	4°C
7	64.2	54.9	86.4	90.5
14	27.9	49.2	83.7	89.0
28	10.0	20.1	53.8	84.0
35	7.7	16.7	51.2	81.3

Table 6 shows the number and percent of water samples which yielded pathogenic Naegleria as a function of temperature in the thermally enriched and three suspect lakes. The seven positive samples from thermally enriched Lake Monroe were obtained in April, March, October, November and December, whereas positive samples from the natural lakes were obtained from June through mid-October with the majority in July and August, 24 and 33 positive samples respectively. (Tables A-1 through A-4, Appendix A). In May when the water temperature in Lake Monroe was 30°C, none of ten ≤2 liter samples were positive. Conversely, when a comparable water temperature was recorded in Lake Hourglass, four of five 0.1 liter samples yielded pathogenic Naegleria isolates. As shown on Table 6, when water temperatures in the natural lakes were between 25°C and 34°C the number of positive specimens equaled or far exceeded those of the thermally enriched lakes, thus indicating lower population levels in the latter. Supportive evidence for this is discussed below. Because the purpose of this study was to determine the distribution of pathogenic Naegleria in Florida's lakes, extensive evaluation of the thermally enriched lake was not undertaken. However, it would be of interest to quantitatively follow pathogenic Naegleria population fluctuations in thermally enriched lakes to determine the real effect of temperature changes due to

thermal cooling water discharges under natural conditions.

TABLE 6. PERCENT OF WATER SAMPLES YIELDING PATHOGENIC NAEGLERIA ISOLATES FROM A THERMALLY ENRICHED AND THREE NATURAL LAKES AS A FUNCTION OF TEMPERATURE

Lakes / Temperature °C	Percent of water samples positive			
	Monroe (thermally enriched)	Conway	Hourglass	Baldwin
20-24	33.3 (2/6)*	0.0 (0/0)	0.0 (0/0)	100.0 (1/1)
25-29	8.3 (2/24)	7.1 (1/14)	15.4 (2/13)	0.0 (0/18)
30-34	14.3 (2/14)	20.0 (6/32)	68.3 (28/41)	85.3 (29/34)
35-39	20.0 (1/5)	0.0 (0/0)	0.0 (0/0)	0.0 (0/0)

* Number of samples positive/number tested.

As previously discussed, Corner Lake, which is comparable in size to Lake Spier, has yielded no Naegleria isolates. Although the lake has been rather extensively studied between October and May (140 samples), findings during hot summer months should be more conclusive. Lake Beauclaire has yielded only a single isolate from a depth of 4 m. Lake Jessup and Tohopekalig have been sampled less extensively but no pathogenic Naegleria have been isolated. These findings may be misleading since even frequent grab samples may result in negative findings if concentration of the agent is at a very low level. As can be noted on Table A-5 (Appendix A), no pathogens were isolated from Lake Spier during 1976, even though sampling took place during the summer months. In May, 1977, an isolate was obtained from the water column when the water temperature was 26°C. Only one plate of 20 inoculated was positive indicating a very low population level. Severe draught conditions in the spring of 1977, caused a drop of two to three feet in lake water levels. This resulted in a natural concentration of lake waters and probably facilitated the isolation of pathogenic Naegleria from the heretofore "negative" Lake Spier. At no time have the numbers of isolates per sample approached those found in samples from Lakes Conway, Hourglass, or Baldwin, indicating the persistence of a relatively low pathogenic Naegleria population level in Lake Spier.

CHARACTERIZATION OF SEROPOSITIVE NONPATHOGENIC AND PATHOGENIC NAEGLERIA

An important aspect of this present study has been the elucidation of the physiologic and antigenic differences between the nonpathogenic and pathogenic Naegleria trophozoites and structural differences between their cyst forms. Two of the early, definitive characteristics of pathogenic Naegleria outgrowth from a field specimen, were the plaque size and the leading edge. Their plaques were smaller than those shown by seropositive nonpathogenic Naegleria. The advancing plaque line was remarkably clean and

appeared to the naked eye as a line drawn on glass by a diamond marking pen. When observed under the microscope, all amoebae appeared to be advancing en masse. Conversely, the seropositive nonpathogenic Naegleria plaque was larger and the advancing plaque line fuzzy to the naked eye. When viewed under the microscope the fuzzy line was seen to be due to individual amoebae advancing before the main plaque line.

The difference in plaque size would lead one to think that the generation time for seropositive nonpathogenic Naegleria would be less than that for pathogens. To determine the validity of this assumption a generation time study was conducted. Eight plaque count plates for each of four strains were seeded with not more than 20 amoebae. Microscopic examination of each plate permitted the selection of an isolated amoeba which was designated as the test organism. The amoeba site was marked on the underside of the plate with a wax crayon. Four of each species of plaque count plates were incubated at 37°C and the remainder at 43°C. Counts were made every two hours through the tenth hour. Results are shown in Table 7.

The difference in generation time is more pronounced at the 43°C temperature than at 37°C. This is in keeping with the higher incubation temperature favored by the pathogenic strain. However, there was an almost two-fold difference in the population levels at 43°C and 37°C, even for the nonpathogens. As had been noted with field isolates, plaques formed by the nonpathogens were larger, i.e., trophozoites dispersed over a larger area by the sixth hour, whereas the pathogenic Naegleria trophozites remained aggregated through the tenth hour. Both reproduction and movement are energy demanding. Why the seropositive nonpathogenic Naegleria utilizes much of its energy in motility as opposed to reproduction and the pathogenic Naegleria does the converse is unclear. However, these data do indicate a distinct physiological difference between the two species.

TABLE 7. GENERATION TIME STUDY OF SEROPOSITIVE NONPATHOGENIC AND PATHOGENIC NAEGLERIA AT TWO TEMPERATURES

Strain	Temperature (°C)	Average plaque counts at time (hours) 0	2	4	6	8	10	Plaque morphology
43-9*	37	1	1.75	4.0	7.75	17.25	39.50	Widely dispersed by t = 8 h
83-16**	37	1	2.25	6.65	13.00	26.50	63.25	Aggregated through t = 10 h
43-9	43	1	2.00	6.50	17.50	30.50	56.50	Widely dispersed by t = 6 h
83-16	43	1	2.25	8.00	24.50	54.50	127.00	Aggregated through t = 10 h

* Seropositive nonpathogenic Naegleria isolated from lake bottom sediments on 2-3-76.

** Pathogenic Naegleria isolated from lake bottom sediments on 2-24-76.

Antigenic differences between the seropositive nonpathogenic and pathogenic Naegleria also have been documented, as well as their antigenic dissimilarity with N. gruberi. Rabbit antisera prepared against the GJ strain were successfully absorbed with the 43-9 strain, the nonpathogenic seropositive lake isolate. Table 8 shows the results of the initial test with this antiserum. With unabsorbed antiserum, N. gruberi (381-2) is readily distinguished from both the pathogenic (GJ and 375-17) and nonpathogenic (43-9) strains since it fluoresced poorly and reached a titer of only 1:40. However, the pathogenic and nonpathogenic strains were positive to within one dilution of each other, 1:1280 and 1:640, respectively. In the presence of rabbit anti-GJ serum absorbed with 43-9 amoebae, the titer against GJ amoebae remained unchanged and against 43-9 amoebae only dropped to 1:40, indicating incomplete absorption. In later tests using completely absorbed 43-9 and GJ rabbit antisera, no heterologous reactions occurred. The homologous titers with these antisera before and after absorption dropped from 1:320 to 1:40 for 43-9 and from 1:640 to 1:160 for GJ.(14) Based on the cross absorption studies, it appears that the specific antigens are minor in the 43-9 strain and major in the GJ, possibly reflecting the complex enzymatic system required for cytolysis which permits tissue destruction by the pathogen.

TABLE 8. INDIRECT FLUORESCENT ANTIBODY TEST RESULTS USING VARIOUS ANTIGENS AND ANTISERA - SEPTEMBER 23, 1976

		Titers*							
Antigen	Antiserum	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
GJ**	GJ #3†	4	4	3+	3	2	1	1	±
375-17††	GJ #3	4+	4	4	3	2	1+	1	±
381-2§	GJ #3	2	1	-	-	-	-	-	-
GJ	GJ #7 & 8§§	4	3	3	2+	2	1	1	±
43-9‡	GJ #7 & 8	3	4	3	2	1	1	±	-
GJ	GJ #7 & 8 (absorbed)‡‡	4	3	2+	2	2	1	1	±
43-9	GJ #7 & 8 (absorbed	2	1	-	-	-	-	-	-
Controls									
GJ	None	-	-	-	-	-	-	-	-
375-17	None	-	-	-	-	-	-	-	-
381-2	None	-	-	-	-	-	-	-	-

* Titers scored on a scale of 1 to 4 based on intensity and number of fluorescent positive sites per slide.
** Pathogenic Naegleria isolated from fatal human case in July, 1973.
† Rabbit anti-GJ serum ampouled on 12-12-75.
†† Pathogenic Naegleria isolated from lake water on 9-9-76.
§ N. gruberi isolated from lake sediments on 9-13-76.
§§ Rabbit anti-GJ serum ampouled on 5-27-76.
‡ Seropositive nonpathogenic Naegleria isolated from lake sediments on 2-24-76.
‡‡ Absorbed with an equal volume of 43-9 amoeba.

Early observations of cysts of the seropositive nonpathogenic and the pathogenic Naegleria indicated that the latter had few if any pores and appeared to have a thinner cyst wall. During sonication of a trophozoite suspension, it was noted that the few cysts present in the 43-9 (seropositive nonpathogen) preparation required a longer exposure time to the shearing forces in order to rupture than did cysts in the GJ (pathogenic Naegleria) preparation. Thus, an experiment was performed to determine the validity of this observation and the magnitude of the difference.

Amoebae from each strain were grown in an inorganic solution containing 2.5% bacterial homogenate of E. aerogenes. After seven days at 43°C, cysts were harvested by centrifugation at 1,000 X g and washed twice with PBS. Final cyst concentrations were adjusted to within 10% of each other (1.42 X 10^6/ml for strain 43-9 and 1.32 X 10^6/ml for strain GJ) as determined by triplicate hemocytometer counts.

Aliquots (15 ml) of each suspension were subjected to sonication in a Branson Sonifier Cell Disruptor, Mdl. No. W-350, at 100 watts using the No. 419 microtip. After 45 seconds an aliquot was removed and the cysts counted. Additional aliquots were removed and counted at 60, 80, 100, and 120 seconds. The following day duplicate aliquots (15 ml) were treated and sampled at various times. Results are shown in Table 9. The sonication time required to disrupt 50% (DT_{50}) of the pathogenic GJ strain cysts was approximately 65 seconds, whereas the DT_{50} for the 43-9 cysts was approximately 100 seconds. Therefore, our data appear to indicate that the seropositive nonpathogenic Naegleria cysts are more resistant to sonication than the pathogenic cysts. Because of the difficulty in distinguishing cyst stages from late pre-cyst stages by low power microscopic examination, there may have been a small percentage of the latter included in the cyst preparations tested. However, this would have been true for both populations because the same individual counted all the cysts. Therefore, it was assumed that such a variable would be held constant. To determine the validity of that assumption, cyst cultures will be subjected to sonic disruption on days 6, 9, 12 and 15. If the results are comparable to those of the initial study, the assumption will be validated.

TABLE 9. COMPARISON OF SHEARING FORCES REQUIRED TO RUPTURE CYSTS OF THE SEROPOSITIVE NONPATHOGENIC AND THE PATHOGENIC NAEGLERIA

	Intact cysts remaining after sonication (seconds)											
Strain	0	45	55	60	65	70	75	80	90	100	105	120
43-9												
Number	142	102.5	--	98.5	--	94.0	90.0	89.0	77.0	72.0	68.0	66.0
Percent	100	72.2	--	69.4	--	66.2	63.4	62.7	54.2	50.7	47.9	46.5
GJ												
Number	132	78	72	69.5	--	63.0	--	57.0	--	45.0	--	36.0
Percent	100	59.1	54.5	52.6	--	47.7	--	43.2	--	34.1	--	27.3

The foregoing data which are summarized in Table A-12 (Appendix A), appear to support the contention that the seropositive nonpathogenic Naegleria is grossly different from the pathogenic strain. When these differences are added to the inability of the nonpathogenic strain to produce fatalities in mice following intranasal instillation of large numbers of trophozoites, it would appear that the differences are sufficiently great to warrant separate species classification for these two agents. As has been suggested previously, the seropositive nonpathogen might well be referred to as Naegleria fowleri and the pathogen as Naegleria invadens.(14) Thus, the species designations would clearly differentiate the pathogen from the nonpathogen.

ANAEROBIC STUDIES

Sixteen IA plates were inoculated with an agar plug from either an actively growing or encysted culture of pathogenic strains GJ and 437-2 (field isolate), N. gruberi 371-2 and the seropositive nonpathogenic 43-9. A cyst and a trophozoite culture plate of each species were placed in an anaerobic jar (Brewer) containing a gas pack (Gas Pak Disposable Hydrogen + CO_2 Generator Envelope, BBL #70304) and methylene blue indicator strips (Gas Pak Disposable Anaerobic Indicators, BBL, Cockeysville, Md.) and incubated at 35°C. Companion plates of each species were incubated aerobically in the same incubator. After 48 hours, the anaerobic jar and the plates were maintained at room temperature for three weeks. The anaerobic jar was then opened and all 16 plates placed in the 35°C incubator and held for three days, at which time cultures were examined for evidence of growth.

The four trophozoite cultures held aerobically all supported trophozoite growth throughout the experimental period. Three of the aerobic cyst cultures showed excystation and heavy trophozoite growth. However, 437-2 cysts failed to excyst and no trophozoite growth resulted.

Under anaerobic conditions, several of the 371-2 trophozoites encysted. When they were removed from the jar and incubated at 35°C, excystation and growth ensued. A few trophozoites of strain 437-2 also encysted but failed to excyst when placed under aerobic conditions at 35°C. Neither 43-9 nor GJ showed any encystment under anaerobic conditions.

Three companion cyst culture strains held anaerobically survived. They excysted and reproduced under aerobic conditions at 35°C. The one exception was strain 437-2. Actually no cysts were visible after the three week anaerobic incubation period. The fact that even under aerobic conditions cysts failed to excyst would appear to indicate an abnormality or nonviability in the cysts of strain 437-2.

These data appear to indicate that seropositive nonpathogenic and pathogenic Naegleria cysts can survive under anaerobic conditions. Therefore, if cysts were to become embedded in anaerobic lake bottom sediments during winter months, they should survive. However, this would preclude their being demonstrated in the water column and perhaps in sediment samples as well, unless sufficient sediment samples were tested. Such negative findings would not indicate the disappearance of pathogenic Naegleria from the lake, but

simply reflect inadequate sampling. As the temperature of the waters increases and the activity in the lake results in agitation of the sediments, the embedded cysts could be freed to initiate the cycle over another summer.

MOST PROBABLE NUMBER (MPN) TEST

Quantitation of pathogenic Naegleria in the water column was initially based on small measured samples; 25, 50, 100, and 200 ml samples were filtered through a cellulose nitrate membrane with an average pore size of 5 μm. Membranes were inverted onto IA plates seeded with E. aerogenes. Because plaques coalesced, it was impossible to obtain accurate counts. Therefore, quantitative results were expressed as one or more pathogenic Naegleria per unit volume.

In an attempt to refine the data, the MPN methodology routinely used in bacteriology was applied to the enumeration of pathogenic Naegleria. A suspension of trophozoites was prepared by washing a plate culture of the GJ strain with PBS (0.4 percent NaCl). The trophozoite concentration was determined by triplicate hemocytometer counts. Appropriate dilutions were made to achieve suspensions of approximately 100 per ml and one per ml. The former was titrated on IA with E. aerogenes using the MPN methodology at 10^0, 10^{-1}, 10^{-2} and 10^{-3} dilutions with five plates per dilution. From the suspension containing one trophozoite per ml, five 100 ml, five 10 ml, five 1 ml, and five 0.1 ml aliquots were filtered through 5 μm porosity cellulose nitrate membranes at approximately 5 psi. Each membrane was inverted onto IA plates seeded with E. aerogenes (Table 10). The results were sufficiently encouraging to warrant field trials.

TABLE 10. MOST PROBABLE NUMBER TEST RESULTS

Test method	MPN code	Index per ml	Expected per ml
Titrated	5,5,3,2	140	100
Filtered	5,5,3,2	1.40	1.0

Three of the suspect lakes were included in the field trial. Water samples were collected in silicone coated, one liter screw cap bottles at depths of 0.3 m, 0.6 m, and 2.4 m from each of the three lakes. Samples were transported to the laboratory and processed within six hours. From each liter sample, five each of 1 ml, 10 ml, and 100 ml subsamples were filtered through 5 μm porosity membranes, the filter removed and inverted onto a seeded IA plate and incubated at 43°C. Amoebae from plaques produced were tested for flagellation and if positive, transferred to CSYECM. Luxuriant growth in this medium was considered a confirmation of pathogenic Naegleria.

Values shown on Table 11 were derived from the standard MPN tables. The relatively high number of positive specimens for Lake Baldwin shown by this

method is comparable to that obtained in the summer of 1976 when at least one pathogenic Naegleria was present in each 25 ml of lake water tested. The findings at Lake Conway were much lower in 1977 using the MPN test than the year before when the small measured samples were used. However, in 1976 the water temperature was 33°C and in 1977, it was 29°C, which may help to account for the lower population. These studies indicated that the MPN methodology could be a useful tool for determining pathogenic Naegleria concentration in lake waters.

TABLE 11. MOST PROBABLE NUMBER STUDIES

Lake	Water temperature (°C)	Sample depth (meters)	Date	Number of positive plates with inoculum (ml) 100	10	1	MPN index liter
Conway	30.0	<0.3	07-22-77	0	0	0	<2.0
		0.6	07-22-77	0	0	0	<2.0
		0.6	07-22-77	0	0	0	<2.0
		2.1	07-22-77	0	0	0	<2.0
	29.0	<0.3	08-29-77	2	2	0	9.0
		<0.3	08-29-77	0	0	0	<2.0
		0.6	08-29-77	0	0	0	<2.0
		0.6	08-29-77	0	0	0	<2.0
		2.4	08-29-77	1	0	0	2.0
Baldwin	30.5	<0.3	08-01-77	5	3	0	79.0
		0.6	08-01-77	5	2	0	49.0
			08-01-77	0	0	0	<2.0
		2.4	08-01-77	5	5	0	240.0
	30.0		08-26-77	5	4	0	130.0
			08-26-77	5	3	0	79.0
			08-26-77	5	5	0	240.0
			08-26-77	5	4	1	170.0
			08-26-77	5	2	0	49.0
			08-26-77	5	2	0	49.0
Spier	31.0	<0.3	08-01-77	2	0	1	7.0
		0.6	08-01-77	0	0	0	<2.0

In a controlled experiment designed to assess whether a direct plaque count could be used instead of the MPN test to determine population sizes, it was noted that an amoebic suspension filtered through a 5 μm porosity cellulose nitrate membrane failed to yield plaques when the membrane was inverted onto an IA plate seeded with E. aerogenes. Thus, the following experiment was carried out to recheck the MPN technique.

A 48-hour culture of a pathogenic field strain was washed from an IA plate and the concentration established by duplicate hemocytometer counts. The concentrate was diluted to approximately 1,000 cells per ml and two sets

of 10^0, 10^{-1}, 10^{-2}, and 10^{-3} dilutions were made. From Set No. 1, five 1 ml aliquots of each dilution were inoculated directly onto individual IA plates seeded with E. aerogenes. From Set No. 2, five 1 ml aliquots of each dilution were filtered through individual 5 µm porosity membranes which were rinsed with distilled water and inverted onto seeded IA plates. From the suspension containing 10 amoebae/ml, five 100 ml, five 10 ml, five 1 ml, and five 0.1 ml aliquots were filtered and plated as described above

On day 14, only Set No. 1 showed any correlation with the initial inoculum. Due to the low counts obtained on the filtered samples, it was hypothesized that trophozoites had been trapped within the membranes and the IA plates were too dry to permit capillary attraction to draw bacteria up into these areas, therefore the trophozoites had encysted due to lack of nourishment. To test this, 0.2 ml of the bacterial suspension used to seed the IA plates was placed on each membrane and the plates reincubated at 43°C for an additional week. Results shown in the last column on Table 12 confirmed the above hypothesis.

Conditions of the initial test, in which good correlation was noted, were revised and it was found that the plates had been freshly prepared and thus contained sufficient moisture to have permitted growth within the interstices. Thus, 0.2 ml of the bacterial suspension is now routinely placed on each membrane when plated.

TABLE 12. MOST PROBABLE NUMBER TEST RESULTS BEFORE AND AFTER* THE ADDITION OF A BACTERIAL SUSPENSION TO MEMBRANES

Set No.	Direct cell count (ml)	Dilutions used	Day 14 Number of positive plates	Day 14 MPN ml	Day 21 Number of positive plates	Day 21 MPN ml
1	1,000	10^0 10^{-1} 10^{-2} 10^{-3}	5,5,5,4	1,600	--	--
2	1,000	10^0 10^{-1} 10^{-2} 10^{-3}	5,2,1,0	7.0	5,5,5,5	>2,400
3	10	10^2 10^1 10^0 10^{-1}	5,4,1,0	0.17	5,5,5,4	16.0

*Bacterial suspension added at day 14 and read seven days later.

STANDARD METHOD DEVELOPMENT

The development of a standard method for isolating amoeba from specimens mailed to a laboratory was deemed necessary so that results would be comparable. Such a method must designate the type(s) of specimen(s) to be obtained and how they are to be handled before and during transport to a laboratory. Because pathogenic Naegleria were isolated from sediment samples and not water samples shipped to ERC, studies were conducted to determine what if any procedures could enhance the isolation rate.

A large sample of bottom sediment was obtained from Lake Conway on March 20, 1978. This was homogenized and eighteen 200 ml subsamples were taken from the large sample and assigned to six groups of three samples. Each group was incubated at room temperature (26°C) or at 43°C for 18 or 96 hours and then either processed immediately or held at room temperature for varying time periods before processing. Each sample was processed individually and plated onto 10 IA plates seeded with E. aerogenes. Thus, 30 plates were examined for each treatment regime. Trophozoites from each plaque were tested for flagellation. Those undergoing flagellate transformation were inoculated into CSYECM. Non-flagellating amoebae were identified by examination of both trophozoites and cysts.

Results are shown on Table 13. The most striking finding was the pure culture of pathogenic Naegleria demonstrated in the samples which had been incubated for 96 hours and then processed immediately. When comparable samples incubated for 96 hours at 43°C were permitted to remain at room temperature for 96 hours before processing, N. gruberi and Hartmanella species were demonstrated. Even so, only about 30 percent of the pathogenic Naegleria were lost over the four-day period, which might be similar to the time required for shipping samples from distant sites to the laboratory. There was little difference between samples held for 18 hours either at room temperature or at 43°C before processing. However, when samples were incubated for 18 hours at 43°C and then held at room temperature for 72 or 168 hours, N. gruberi became the predominant isolate, i.e., 83.3% and 78.6% at each of the respective temperatures. These data demonstrate that proliferation of N. gruberi is favored when the sediments are held at room temperature.

Based on these data, all sediment samples are held at 43°C for 96 hours before processing. No quantitative data can be derived from these enriched specimens, but the probability of isolating pathogenic Naegleria is definitely enhanced. This is extremely important when specimens from suspect lakes are to be examined in a laboratory located hundreds of miles away from the collecting site. This procedure also extends the time required to confirm the presence of pathogenic Naegleria in a suspect exposure site. The enhanced probability of making an isolate using this procedure far outweighs the disadvantage of delay. However, if a rapid answer were desired, additional sediment samples could be examined without preincubation but negative findings would need to be confirmed by processing the companion samples which had been incubated.

Figure 4 depicts the laboratory tests employed and the time usually required for processing specimens. Once specimens are processed and incubated, a presumptive positive identification can be made in three to four days based on the appearance of the plaque line and luxuriant growth in CSYECM. Confirmation in mice requires at least four additional days.

Amoebae from spinal fluids, in case of human disease, could be identified by IFA studies within a few hours if sufficient trophozoites were present. Cerebral spinal fluids obtained aseptically could be inoculated directly into CSYECM for shipment to a laboratory. Incubation of the suspension at 37°C for at least 18 hours would enhance the probability of survival during shipment.

TABLE 13. ISOLATION RESULTS FROM SIX INCUBATION TREATMENTS OF SEDIMENT SAMPLES

Treatment*			Number plaques positive for						
No. hours at 43°C	No. hours at room temp.	Number of plaques**	Flagellation	Growth in CSYECM	Number identified as P.N.†	N.G.††	Hart.§	Vah.§§	% Pathogenic Naegleria
0	18	21	10	6	6	4	9	2	28.6
18	0	28	13	11	11	2	12	3	39.3
18	72	30	27	2	2	25	1	2	6.7
18	168	28	24	2	2	22	1	2	7.1
96	0	30	30	30	30	0	0	0	100.0
96	96	31	28	22	22	6	3	0	71.0

* Three replicate samples per treatment.
** Plaques from 30 plates inoculated, i.e., ten for each replicate sample.
† Pathogenic Naegleria.
†† N. gruberi.
§ Hartmanella sp.
§§ Vahlkampfia sp.

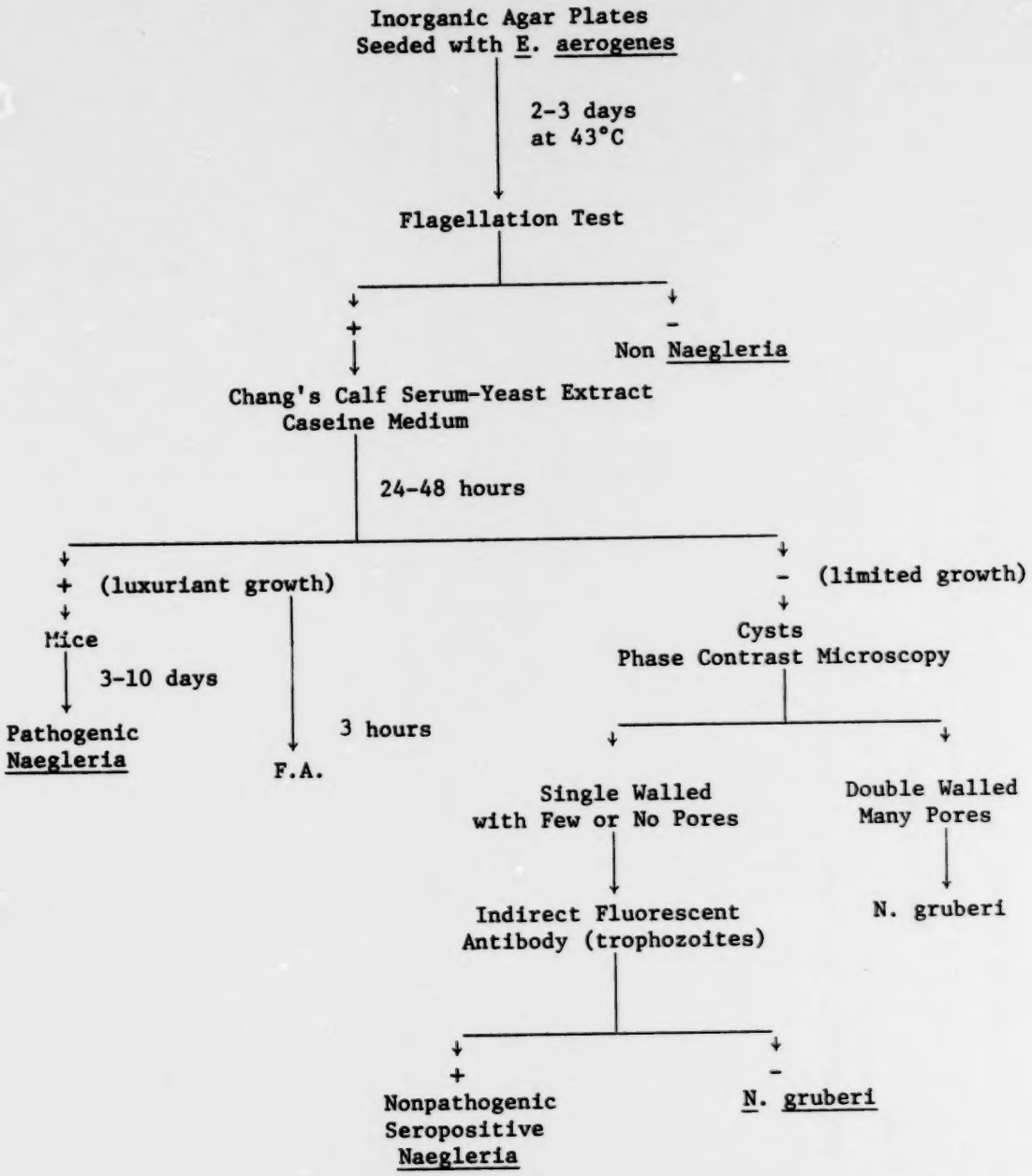

Figure 4. Flow sheet and time required for isolation and identification of pathogenic Naegleria from field specimens.

MINIMAL LETHAL DOSE EXPERIMENTS

From an epidemiological standpoint, knowing the minimal mouse lethal dose (MLD) would be most important. In a preliminary experiment, a single pathogenic Naegleria trophozoite inoculated into four tubes of CSYECM produced an amoeba population in three of the cultures. Therefore, under ideal circumstances a single trophozoite would be necessary to initiate an infection in vivo. In view of the numerous variables involved in intranasal (IN) infections, one would not anticipate the same dose response. Therefore, an attempt was made to determine the MLD in mice following IN instillation of the organism.

A suspension containing 100,000 trophozoites per ml was prepared based on triplicate hemocytometer counts using pathogenic strain 76N-437 which had been isolated from Lake Bell on October 11, 1976. Its passage history was: initial isolation on IA, one passage in mouse brain, 18 in CSYECM, two on IA, one in mouse brain, and five on IA. Thus, in roughly 20 weeks this isolate had been passaged 28 times before being used in the MLD experiment. Two-fold dilutions of the suspension were made and carried well beyond the endpoint. When the initial suspension was back titrated, the original count was found to be valid. Table 14 (see p. 34) details the initial inoculation and subsequent challenge of survivors. No deaths occurred with less than 78 trophozoites, and only two of five mice succumbed as a result of this dose. Deaths were erratic and even 2,500 trophozoites failed to produce death in all mice inoculated. When survivors were challenged with twice the maximal initial dose, the same irregular death pattern occurred. The challenge suspension was prepared from the 76N-437 culture which had undergone two additional passages on IA. As shown in Table 15 and Figure 5, incubation periods were extended beyond day 12 in 12.2% of the mice and flaccid paralysis of the posterior limbs developed on day 26 in one animal. The latter was sacrificed and the brain tissue inoculated into CSYECM and onto IA plates seeded with E. aerogenes.

TABLE 15. PERCENTAGE OF DEATHS ON INDIVIDUAL DAYS POST INOCULATION: INITIALLY AND AFTER CHALLENGE

	Percentage of deaths on post inoculation days									
Inoculation	5	6	7	8	9	10	12	14	16	19	26
Initial		35.7	14.3		21.4	7.2	21.4				
Challenge	2.0	38.8	18.4	8.2	8.2		12.2	4.1	2.0	4.1	2.0

Few amoebae were noted after two days in CSYECM. Usually, brain tissue derived from mice that succumbed to pathogenic Naegleria infections showed extensive amoebic growth within twenty-four hours after inoculation into CSYECM. Only three plaques developed on the IA plates; one with a clear, sharp plaque line typical of pathogenic Naegleria, one with a "fuzzy" plaque

TABLE 14. DATA ON MOUSE MINIMAL LETHAL DOSE AND CHALLENGE EXPERIMENT

		Initial inoculation				Challenge with 5×10^3 trophozoites			
Date of Inoculation	Lab No.	No. of amoeba	No. of test animals	No. of survivors	Percent survivors	Date	No. of test animals	No. of survivors	Percent survivors
3-9-77	317	0.035	5	5	100	3-30-77	5	2	40
	316	0.07	5	5	100		5	0	0
	315	0.15	5	5	100		5	3	60
	314	0.30	5	5	100		5	1	20
	313	0.60	5	5	100		5	1	20
	312	1.2	5	5	100		5	0	0
	311	2.4	5	5	100		5	0*	0
	310	4.9	5	5	100		5	3	60
	309	9.8	5	5	100		5	2	40
	308	19.5	5	5	100		5	1**	20
	307	39.1	5	5	100		5	1	20
	306	78.1	5	3	60		3	0	0
	305	156.2	5	4***	80		3	2	66.6
	304	312.5	5	2	40		2	2	100
	303	625.0	5	4	80		4	3	75
	302	1,250.0	5	2	40		2	0	0
	301	2,500.0	5	1	10		1	0	0
	Total		85	71	83.5		70	21	30

* 4th death occurred on day 9 and the carcass was eaten by litter mate who died three days later on day 12.

** On day 26, one of two remaining litter mates developed posterior flaccid paralysis, was sacrificed and very few amoebae were demonstrated by inoculation of brain into CSYECM and inorganic agar media.

*** One animal died at time of challenge.

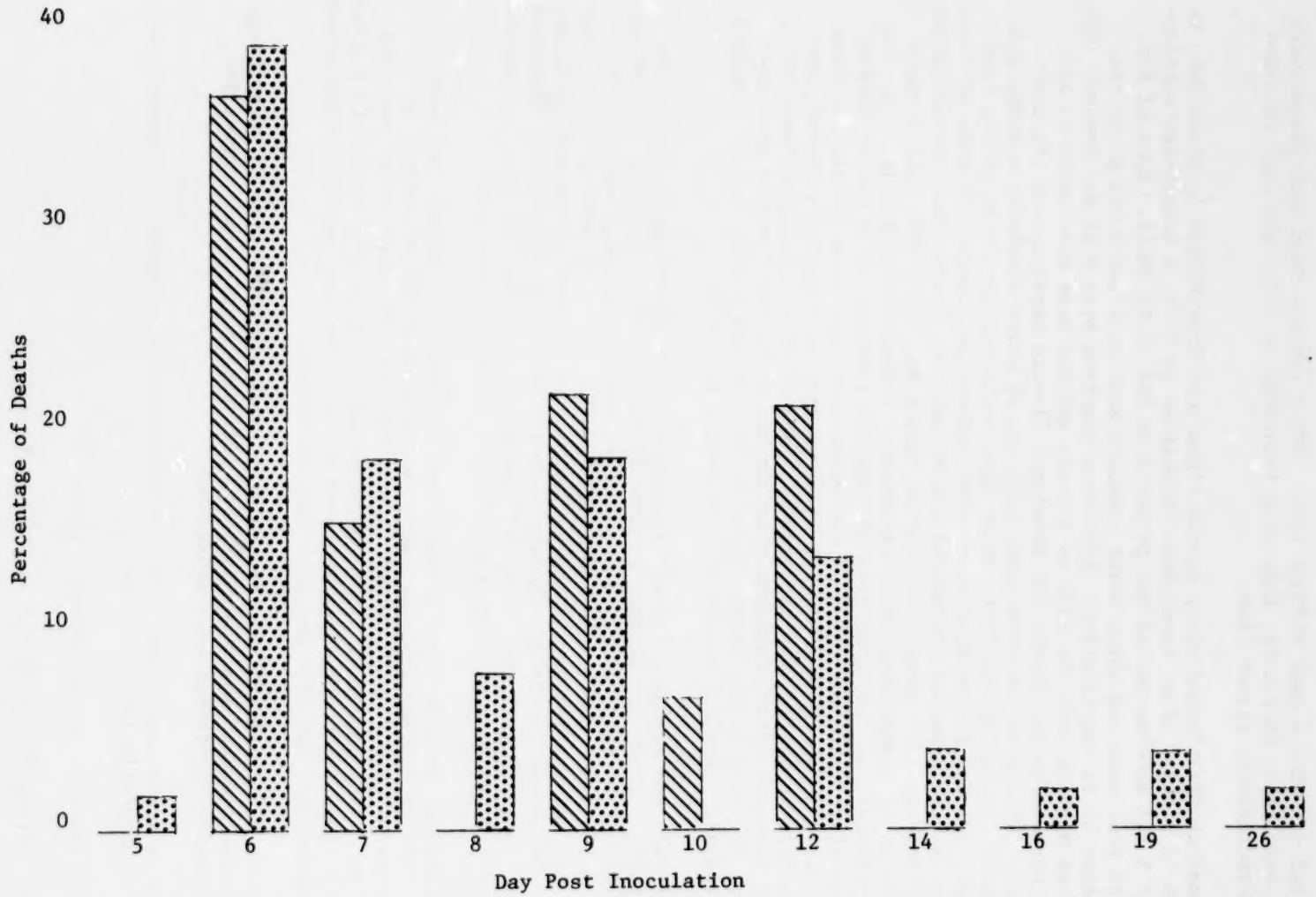

Figure 5. Percentage of mouse deaths on individual days post inoculation and after challenge with 5,000 trophozoites instilled intranasally. ▨ inoculation; ▩ challenge.

line, and one with a more diffuse line. After progeny from each plaque were passed onto fresh IA plates, they were incubated at 37°C, and each retained its characteristic plaque line.

Amoebae that formed sharp plaque lines were transferred in three days to a fresh IA plate. After three days incubation at 37°C, a suspension containing 4.1 X 10^5 amoebae per ml was prepared in PBS (0.4% NaCl). Each of six, three to four week old white Swiss weanling mice received 0.025 ml of the suspension by IN instillation. Mice were observed over a 14 day period. One mouse was missing from the cage on day six and may have been eaten by its litter mates. On day seven, one developed flaccid paralysis of its hind quarters. On day 10 two mice died, only one of which displayed a comparable paralysis. On day 12 the fifth mouse died following paralysis of the hind quarters, and day 14 the last mouse died without paralysis. In none of these animals was the expected cerebral edema noted. All brains were harvested and yielded amoebae when inoculated onto IA plates and in CSYECM. All plaques produced on IA plates were characteristic of pathogenic Naegleria, i.e., had sharp plaque lines. Progeny from the amoebae isolated on IA and in CSYECM from the first brain harvested on day seven were instilled IN into weanling mice. Four of four mice receiving IA grown amoebae died on day seven, and amoebae were recovered from the brains. Five of five mice which received progeny from the CSYECM cultures died but the incubation periods were extended. One died on day seven, three on day ten and one on day 20. Amoebae were recovered from all brains in both CSYECM and on IA.

IA cultures having a "fuzzy" plaque line were passed on IA weekly for eight weeks. From that passage an amoebic suspension of 2.7 X 10^5 per ml was prepared in PBS (0.4% NaCl). Each of five, white Swiss weanling mice received 0.025 ml of the suspension instilled IN. Two mice died on day 15, one on day 16, and one was sacrificed on day 21 when posterior limb paralysis was noted. One mouse survived. Amoebae were isolated from each of the harvested brains. Isolates grew well in CSYECM and showed a 1:640 IFA titer but plaque lines were sharp as opposed to the original "fuzzy" plaque line.

After ten IA transfers, progeny from the amoebae showing a diffuse plaque line on initial isolation were instilled in 0.025 ml amounts at a concentration of 2.9 X 10^5 per ml IN into each of five white Swiss weanling mice. No deaths occurred. These amoebae had failed to grow in CSYECM but had shown the same 1:640 IFA titer achieved by the other two isolates which proved to be pathogenic for mice and grew well in CSYECM.

In an effort to explain the isolation from a single infected mouse brain of a nonpathogen and two other isolates differing in virulence expression, the following hypotheses were considered:

1) a mixed population, i.e., seropositive nonpathogenic and pathogenic Naegleria in the culture

2) transfer factor and/or a virus

3) strain selection due to culture techniques

4) immunological status of mice

The following studies were conducted to elucidate these possibilities.

Although a mixed culture of seropositive nonpathogens and pathogenic Naegleria was unlikely because of the differences in their plaque characteristics, the possibility was investigated. A suspension containing both was prepared and instilled IN into mice.

A pathogen isolated from lake water, 77N-795 and a nonpathogen, 43-9, were washed with PBS (0.4 NaCl) and three suspensions prepared as follows. The number of pathogens was held constant at 10^6 per ml in all instances. Nonpathogens were at levels of 2.5×10^5, 1.25×10^5 and 6.25×10^4 per ml in suspensions 1, 2, and 3, respectively (see Table 16). Groups of ten, three-to four-week-old white Swiss weanling mice received 0.005 ml IN from one of the three suspensions. Mice were observed daily for morbidity. All suspensions produced death in 70% to 90% of the mice in five to six days, which is typical of the pathogenic Naegleria. Ill mice were sacrificed and harvested, brain tissues placed on IA cultures at both 37°C and 25°C and plates observed daily for plaques. No isolates of 43-9 (seropositive nonpathogen) were recovered.

Control plates inoculated with the three test suspensions were held at 37°C and room temperature. At both temperatures, the nonpathogens grew out and masked the presence of the pathogens. When plaques were permitted to grow out to the edge of the plate, washings contained only the 43-9 strain based on their failure to multiply after being inoculated into CSYECM. If a mixed culture had been present in the harvested brain tissues, the nonpathogen, 43-9, should have been isolated under the test conditions, i.e., should have overgrown the pathogens. These findings did not support the hypothesis that seropositive, nonpathogenic amoebae isolated from the paralyzed mouse were in the initial inoculum.

TABLE 16. RESULTS OF MOUSE INTRANASAL INOCULATION OF A MIXED CULTURE OF PATHOGENIC AND SEROPOSITIVE NONPATHOGENIC NAEGLERIA

Suspension	No. mice dead/ inoculated	Percent of deaths	Number of trophozoites inoculated	Percent nonpathogenic	Day of death
#1	7/10	70	6,250	25.0	5-6
#2	9/10	90	5,625	12.5	5-6
#3	8/10	80	5,313	6.25	5-6

To investigate the possibility of a transfer factor or a virus, a culture medium exchange experiment was conducted using pathogenic strain 76N-437 and the seropositive nonpathogenic strain 43-9. Thirty ml of CSYECM were placed in each of two 75 cm^2 cell culture flasks. Both were inoculated

with strain 76N-437 and incubated at 37°C for three days at which time approximately 10^6 amoebae per ml were present in each flask based on duplicate hemocytometer counts. The amoeba suspension was removed from one of the flasks, sonicated at 100 watts X 15 minutes in a rosette cooling cell, and filtered through a 0.45 µm porosity cellulose nitrate membrane. The suspension from the second flask was filtered only. Into one of two 75 cm^2 flasks was placed 12 ml of the sonicated filtrate, and into the second flask, 12 ml of the nonsonicated filtrate. Twelve ml of CSYECM and 0.5 ml of a bacterial homogenate were added to each flask to facilitate axenic culture of the nonpathogenic seropositive 43-9 strain which was inoculated into each flask. After five days of incubation at 37°C, aliquots were removed, placed on IA plates, and incubated for subsequent mouse inoculation and IFA study.

None of the progeny produced fatalities in mice following IN instillation nor did IFA titers differ from the control. These data failed to confirm the presence of a transfer factor under the conditions of this experiment. Unfortunately, only a small portion of the amoebic population was tested. Therefore, if only a small portion of the population were affected, which would be expected if either a transfer factor or a virus were involved, the probability of their being detected in the small volume tested is miniscule. The experiment will be repeated and the total population will be inoculated into CSYECM so that even a low-level transfer-positive or virus infected population could be demonstrated. Even so, the negative data accrued does not support the initial hypothesis.

In considering the strain selection theory the MLD experiment was repeated using a field isolate which had been passaged even more often than had the strain used in the first MLD experiment. Strain 77N-475 had been passed twice on IA, twice in CSYECM, once in mice and 13 times on IA. It had been isolated from lake bottom sediments on June 28, 1977, and was used in the MLD experiment on October 3, 1977. Based on 18 passages over a 14 week period, this strain had been passaged an average of every five days.

A suspension of trophozoites at 350,000 per ml was prepared from an IA plate culture and dilutions made as shown on Table 17. Each dilution in quantities of 0.025 ml was instilled IN into each of five, three- to four-week-old white Swiss mice which were observed over a 21 day period. A direct dose response relationship was shown. The MLD of 70 amoebae was comparable to the 78 MLD shown on the first experiment. However, 33 of the 45 mice survived the initial inoculation and were challenged by IN instillation of 10,000 trophozoites of the same strain (77N-475) which had had four additional IA passages in the three week interval. Only 9.1% (4/33) of the mice succumbed to the infection. Because of the low fatality rate produced both initially (26.7%) and following challenge (9.1%), plus the fact that the typically sharp plaque line of earlier passages on IA had been replaced by a more diffuse plaque line, 25 mice were inoculated IN with 0.025 ml of a suspension of trophozoites (strain 77N-475 which had undergone seven additional passages on IA over a four-week period) at a concentration of 4.5×10^5 per ml. Not one fatality occurred among the 25 mice inoculated.

TABLE 17. RESULTS OF MINIMAL LETHAL DOSE AND CHALLENGE EXPERIMENT IN MICE

Initial inoculation (10-3-77)				Challenge with 10,000 amoeba (10-27-77)	
Dilution	No. of amoebae instilled	No. deaths/ No. inoculated	Percent survivors	No. deaths/ No. inoculated	Survivors
10^{0}	8,750	5/5	0	0	0
$10^{-0.7}$	1,750	4/5	20	0/1	100
$10^{-1.4}$	350	2/5	40	0/3	100
$10^{-2.1}$	70	1/5	80	1/4	75
$10^{-2.8}$	14	0/5	100	1/5	80
$10^{-3.5}$	2.8	0/5	100	1/5	80
$10^{-3.8}$	1.4	0/5	100	1/5	80
$10^{-4.1}$	0.7	0/5	100	0/5	100
$10^{-4.4}$	0.35	0/5	100	0/5	100

When 77N-475 was isolated initially, it was a typical pathogenic Naegleria which killed 80 to 90% of the mice in five to six days. Therefore, an earlier passage from stock (passage 9) was inoculated IN into five mice and the high passage 77N-475 which had undergone seven additional passages on IA over a four-week period was inoculated into five additional mice. Each was instilled IN with 0.025 ml of a suspension containing 6.5×10^{5} low passaged trophozoites and 8.5×10^{5} high passaged trophozoites. Four of five mice receiving the former died within 11 days, none of five died after receiving the latter.

It was noted early in the project that repeated transfers of pathogenic Naegleria in CSYECM resulted in an attenuated strain incapable of killing mice following IN instillation. However, when these attenuated strains were administered IC, five of five mice died within six days and the recovered brain passaged strains once again were capable of producing fatal infections by IN instillation. It was also noted that pathogens maintained on IA for long periods of time with passage every 3 to 5 months did not show any decrease in pathogenicity. However, both strains used in the MLD experiments had undergone numerous rapid passages on IA and did show a decrease in mouse pathogenicity comparable to that seen following CSYECM long-term culture.

Both strains, 76N-437 and 77N-475, had been used frequently as positive controls for IFA. When first isolated, they were typical pathogens in that they underwent flagellate transformation, produced sharp plaque lines, grew well in CSYECM, showed a 1:640 IFA titer, and produced death in weanling mice within a five-to seven-day period following IN instillation. Because they

were used as controls, they had been passaged at least weekly and sometimes twice weekly. Since an earlier passage of strain 77N-475, when instilled IN into mice, killed them all and the high passaged strain killed none, it would appear that rapid passage on IA may select for the less virulent strains.

To confirm this, strain 78N-470, a field isolate obtained on June 6, 1978, was passaged approximately every five days on IA, and every third passage, an additional IA plate was inoculated. When grown out, trophozoites were inoculated into mice IN. Table 18 shows the results.

The decrease in virulence as expressed by an increased incubation period is not evidenced until passage 15, when four of five mice died on day seven and one died on day 11. This is much more pronounced by passage 18, when only two of five mice inoculated died, both on day 12. By passage 22, one death occurred on day 18. At the same time, the plaque lines began to be less sharp. Even so, the culture could be maintained in CSYECM, and IFA titers remained constant. These data confirm the selection process resulting from rapid passage on IA.

TABLE 18. DECREASE IN VIRULENCE FOLLOWING RAPID PASSAGE ON INORGANIC AGAR

Date	Passage level (IA)	Trophozoite/ inoculum (0.025 ml)	No. deaths/ No. inoculated	Day of death
6-16-78	3	14,500	5/5	4,4,4,4,4
6-30-78	6	13,750	5/5	4,4,5,5,5
7-14-78	9	14,250	5/5	5,5,5,6,6
7-28-78	12	14,750	5/5	5,5,6,6,6
8-10-78	15	10,750	5/5	7,7,7,7,11
8-24-78	18	11,000	2/5	12,12
9-08-78	22	13,750	1/5	18

Trophozoites do not encyst in CSYECM nor did they encyst during rapid passage on IA. In both cases pathogenicity for mice following intranasal instillation was repressed. Pathogenicity is restored in CSYECM passaged amoebae following either IC inoculation in mice or slow passage on IA which permits encystment. Therefore, it is suggested that pathogenicity for man may reside only in those trophozoites which have undergone recent excystation, which most likely would occur on or near the lake bottom. If this is true, it would support epidemiological data which indicate that infections in man occur most frequently in those individuals who swim underwater, near the lake bottom. Perhaps the mechanism (enzyme?) required for excystation is the same as that required for invasion, i.e., lysis of cell walls. During axenic culture and rapid passage on IA, such an enzyme would not be required;

therefore, it may be repressed. If encystation is permitted the need to excyst when ideal conditions are present may lead to derepression. Cultures that have undergone rapid IA passage will be permitted to encyst in an effort to elucidate this.

It must be emphasized that the seropositive nonpathogenic Naegleria isolated in nature is different from nonpathogenic strains produced by the pathogen. First, the IFA titers of the latter are always the same as the pathogenic parent. The seropositive nonpathogens never achieve a comparable titer. Secondly, and possibly more important, regardless of the inoculation route, IN or IC, the seropositive nonpathogen has never been capable of producing fatalities in mice. Conversely, the avirulent amoebae produced by the pathogenic Naegleria strain quickly revert to virulence following IC inoculation. Lastly, the seropositive nonpathogens cannot be maintained in CSYECM, whereas the avirulent strains derived from axenically cultured or rapidly passaged pathogenic Naegleria can be, although growth of the latter is light compared to that of the parent strain. These data clearly indicate that the two strains are grossly different (see Table A-12, Appendix A).

There is little doubt that variation in susceptibility among mice would play a role in the outcome of an infection with these amoebae. Age, sex, and genetic strain are among the variables. Haggerty, et al.,(15) have elucidated the relative importance of the latter two variables. Mouse experiments conducted during this study did not address these issues. However, since all experiments were conducted in three- to four-week-old white Swiss weanling mice, these two variables were constant. The only exception was the age of mice at challenge. Test groups included both males and females with the overall majority being males. This too was the same in all experiments so that results obtained would appear to be related more to the amoeba per se than to the susceptibility of the mice.

SODIUM CHLORIDE TOLERANCE

Four seropositive nonpathogenic and four pathogenic Naegleria were grown in duplicate cultures on bacteria seeded IA plates containing increasing increments of NaCl from 0.5 to 1%. Concentrations were standardized by counts and 0.025 ml applied to the center of the plate. These were incubated at 35°C for 44 hours when plaque sizes were measured on the duplicate plates and averaged. Table 19 shows the results.

Two of the seropositive nonpathogens, one isolated in Florida and one in Belgium, grew moderately well in the presence of saline at concentrations of 0.5 to 0.9% with their maximal growth at 0.6% saline. A second nonpathogenic Belgian isolate showed little growth at or above 0.85% saline, whereas one local isolate showed very limited growth in the presence of even 0.5% saline and none at higher concentrations.

Three of the pathogenic Naegleria strains grew in the presence of physiological (0.85%) saline but one did not. This was surprising, in that the latter strain, GJ, had been isolated from a fatal case of PAM. Either this strain had been altered during passage or higher saline concentrations can be tolerated in vivo than in vitro. Only one of the pathogens, that isolated

from Lake Okeechobee, produced growth in the presence of 0.9 to 1.0% saline. Unfortunately, the saline content of the lake waters was not ascertained when specimens were obtained. These data would appear to indicate that pathogenic Naegleria would not be found in a salt water milieu. This is in keeping with the epidemiological data indicating exposure to freshwater lakes as a link in the infection chain for PAM.

TABLE 19. PLAQUE SIZE OF SEROPOSITIVE NONPATHOGENIC AND PATHOGENIC NAEGLERIA AS A FUNCTION OF SODIUM CHLORIDE CONCENTRATION

	Average plaque radius at 44 hrs. (mm)							
	Nonpathogenic strains				Pathogenic strains			
%NaCl	43-9*	76-36-250**	LVH_1**	356*	375*	KUL†	GJ††	83*
0.50	17	12	7	<1	14	5	5	3
0.55	25	12	6	NG	14	4	5	4
0.60	25	15	6	NG	12	4	4	4
0.65	21	14	7	NG	12	3	4	3
0.70	16	13	5	NG	8	3	3	2
0.75	16	12	4	NG	7	<1	2	1
0.80	12	11	4	NG	6	<1	NG	1
0.85	12	8	2	NG	5	<1	NG	1
0.90	11	10	1	NG	4	NG	NG	NG
0.95	3	6	NG	NG	3	NG	NG	NG
1.000	4	4	NG	NG	<1	NG	NG	NG

* Florida field isolates.
** Belgian field isolates.
† Belgian human isolate.
†† Florida human isolate.
NG No growth

INTRA-AURICULAR INOCULATION

The Georgia child who died of PAM in 1977 was shown at necropsy to have had a ruptured tympanic membrane. To test the possibility that this was the portal of entry, a guinea pig was inoculated with .02 ml of a suspension containing 3.4×10^5 amoebae/ml in CSYECM. A 22 gauge needle pierced the tympanic membrane and organisms were deposited into the middle ear. The animal's rectal temperature was checked daily for 11 days, after which time the animal was sacrificed and a necropsy done. Several pieces of brain tissue were inoculated onto IA plates and into CSYECM. No elevation in temperature or physical signs of illess were noted over the 11 day observation period. At necropsy, no gross pathology was noted. However, a single CSYECM culture yielded pathogenic Naegleria. This may be significant but most likely resulted from contamination during necropsy. Due to the relative dryness of the cochlea, it is hypothesized that encystment occurred and, thus, may have contaminated one of the tissue specimens at necropsy.

Additional guinea pigs were monitored for basal body temperature for 72

hours prior to being separated into four groups of four animals each. Group 1 was inoculated intracardially with 0.025 ml of a suspension containing 1.0 X 10^6 pathogenic Naegleria per ml (field isolate 78N-198, passage 7). In Group 2, a 20 guage needle attached to the inoculating syringe was pierced through the tympanic membrane and the inoculum deposited in the middle ear. In Group 3, the inoculum was placed directly into the oral cavity and readily swallowed. The inoculum was deposited on the eyes in Group 4. Table 20 shows the results. No infections followed oral or optic inoculation. Deaths following intracardial inoculation were not surprising, since the blood stream permitted widespread dissemination of the organism. Because the deaths due to middle ear inoculation were unexpected, amoebae dissemination via the blood stream due to rupture of the tympanic membrane at the time of inoculation was considered. Therefore, the tympanic membrane was removed from a guinea pig and allowed to heal. A week later, an inoculum containing ca 27,000 trophozoites was deposited in the middle ear. The animal was held on its side to preclude loss of the inoculum. After ca five minutes soft wax was placed in the ear canal and a bandage applied to hold the inoculum in place. No temperature elevation or illness was noted over a 27 day period, therefore it was concluded that deaths in guinea pigs following inoculation through the intact tympanic membrane resulted from hematological dissemination of the pathogenic Naegleria. This rules out the ear as the portal of entry in the Georgia case in which there was evidence of an old tympanic membrane rupture. However, if the tympanic membrane were ruptured while an individual were swimming in pathogenic Naegleria contaminated waters, infection via the circulatory system could conceivably occur.

AXENIC CULTURE

Axenic cultures of both the seropositive nonpathogenic and pathogenic Naegleria were desireable for absorption of antisera and electronmicroscopic comparison studies of the two strains. CSYECM provided prime cultures of the pathogenic strain but an axenic culture medium had to be developed for the seropositive nonpathogens. These data have been published in detail elsewhere(14)but briefly, one liter cultures of E. aerogenes were prepared and incubated at 37°C for four days, after which the bacteria were removed by centrifugation. Cells were resuspended, washed three times and disrupted by sonication in distilled water. One half of the suspension was heat treated to kill any surviving bacteria and the remainder was recentrifuged and filtered through a 0.45 µm porosity cellulose nitrate membrane and designated as cell free lysate (CFL). The heat-treated unfiltered portion was designated bacterial homogenate (BH). These were added in 0.0125 ml increments, 0 to 0.05 ml/ml of CSYECM, Page's malt-yeast extract-amoeba saline medium(16) or inorganic solution. Regardless of the medium used or the quantity of BH or CFL, the seropositive nonpathogenic Naegleria grew fairly well. Maximal growth occurred in CSYECM with 0.05 ml of either BH or CFL per ml of medium. Good growth was noted in the other media at the same supplement concentration. The axenic culture requirements of the two strains accentuates the difference between the nutritional needs of the pathogen and those of the seropositive nonpathogenic Naegleria.

TABLE 20. RESULTS OF PATHOGENIC NAEGLERIA INOCULATIONS OF GUINEA PIGS BY VARIOUS ROUTES

Animal* group - number	Route of inoc.	Days until death	Specimens assayed for pathogenic Naegleria									Comments
			Heart	Lung	Liver	Blood	Brain	Ear	Eye	Tongue	Stomach	
I-1	Intra-cardial	7	+	-	+	+	+	ND†	ND	ND	ND	
I-2	↓	5	+	+	+	-	+	ND	ND	ND	ND	
I-3		11	+	+	+	-	+	ND	ND	ND	ND	
I-4		5	+	+	+	-	+	ND	ND	ND	ND	
II-5	Ear	18	-	+	-	-	+	-	ND	ND	ND	
II-6		4	-	-	-	-	+	-	ND	ND	ND	Only brain tissue next to ear was positive. Animal dead at least 24 hours
II-7		9	+	+	+	+	-	+	ND	ND	ND	
II-8	↓	7	+	+	+	+	+	+	ND	ND	ND	Left & right side brain tissue (+); center tissue (-)
III-9	Oral	Sacrificed at 7 days	-	-	-	-	-	ND	ND	ND	ND	No evidence of illness when sacrificed
III-10		at 14 days	-	-	-	-	-	ND	ND	ND	ND	
III-11		at 21 days	-	-	-	-	-	ND	ND	ND	ND	
III-12	↓	at 28 days	-	-	-	-	-	ND	ND	ND	ND	
IV-13	Eye	at 14 days	-	-	-	-	-	ND	ND	ND	ND	
IV-14		at 7 days	-	-	-	-	-	ND	ND	ND	ND	
IV-15		at 21 days	-	-	-	-	-	ND	ND	ND	ND	
IV-16	↓	at 28 days	-	-	-	-	-	ND	ND	ND	ND	↓

* No temperature elevations noted throughout experiment.
† ND - Not done.

ELECTRON MICROSCOPY

Cysts of the pathogenic Naegleria were disrupted by sonic forces much more rapidly than were cysts of the seropositive nonpathogen. This finding suggested that structural differences in the cyst walls may account for the difference. Therefore, electronmicroscopic (EM) studies were initiated.

Both the seropositive nonpathogenic and the pathogenic Naegleria were grown in CSYECM. Bacterial homogenate was added as a nutritional supplement for the nonpathogens. After two weeks, cysts were harvested and fixed for one hour at 25°C in a mixture of equal volumes of six percent glutaraldehyde and 0.12M sodium cacodylate (NaCac) buffer (pH 6.8). They were rinsed with 0.06M NaCac at 4°C, then centrifuged. This was repeated four times. Cysts were then postfixed for one hour at 4°C in a mixture of osmium tetroxide and NaCac at final concentration of 2% and 0.06M respectively. The rinsing procedure was repeated as before. Following the last rinse, the pellet was embedded in 2% Difco Noble agar.(17) The tissue was minced into small pieces and stained at 4°C with 0.25% uranyl acetate solution (aqueous). A slow-schedule dehydration of the stained pieces was performed using ethyl alcohol followed by several changes of anhydrous acetone before the pieces were embedded in Spurr's(18) low viscosity embedding medium. Thin sections were cut with glass knives on a Sorval Porter-Blum ultramicrotome. The sections were mounted on naked 300 mesh copper grids and stained with uranyl acetate and lead citrate. Electron micrographs were obtained on a Phillips EM200 using Kodak Electron Microscope film.

Unfortunately, none of the prints made of the pathogenic Naegleria cysts showed a complete cross section of a cyst like that obtained for the seropositive nonpathogenic Naegleria (see Figure 6). Partial sections of the pathogenic Naegleria cyst walls did appear to be much less dense than those of the nonpathogens but due to the lack of a good print, such a difference may be an artifact.

Additional studies which actually compare cyst wall thickness should be done. Verification of gross structural differences would increase the list of differences between these two amoeba species.

SUPPORTIVE ACTIVITIES

Suspect Site Investigations

As an adjunct to the surveillance studies conducted the Tampa Epidemiology Research Center (ERC) has served in a supportive role when cases of PAM were diagnosed in humans. Studies done and data accrued are shown below according to the state in which the case was diagnosed.

Georgia

In August, 1977, ERC staff members went to Hephzibah, Georgia, to participate in the investigation of a PAM case involving a 14-year-old black female. She had been swimming at a privately-owned recreational park before the onset of illness. Since the epidemiological evidence pointed to the

Figure 6. Seropositive nonpathogenic Naegleria cysts cross section. Print magnification 10,770.

probability that this pool was the site of exposure, it was the focal point of the investigation.

The swimming pool was constructed with concrete sides and a sandy bottom underlined with clay. Next to the pool was an eight acre lake elevated ca 15 feet above the pool and separated from it by a cement walkway, a terraced area, and a narrow dirt road. A pipe connected the lake and pool which permitted gravity feed from the lake into the pool during high water. A second two acre lake was connected to the eight acre lake in much the same fashion as described above. This produced a "lock" effect with the two acre lake at the highest elevation and the pool at the lowest. Both lakes had natural sand-clay bottoms and sides and were surrounded by sparsely populated, heavily wooded areas. According to local reports, very little swimming was ever done in either lake.

In all, 62 samples were collected. The samples included small and large volumes of water, 100 ml or 200 ml of sediments, and approximately 500 ml of water for the MPN test. The MPN sample in 1 ml to 100 ml aliquots, was filtered on-site using Swinney filter units equipped with 5.0 µm porosity cellulose nitrate membranes (Millipore Corp., Bedford, Mass.). After filtration, membranes were inverted onto IA plates covered with E. aerogenes and allowed to incubate at 43°C for 14 days. Identification of the isolates was based on plaque morphology, flagellate transformation, IFA tests, and growth in CSYECM and was confirmed by mouse intranasal instillation.

Additionally, chemical analysis of the pool water was performed using an engineer's field kit (Mod. DR-EL/2, Hach Chemical Co., Loveland, Co.). Bacteriological analysis (total and fecal coliforms) was performed on the pool waters by the U.S. Army Health and Environmental Activity, Dwight D. Eisenhower Army Medical Center, Ft. Gordon, Ga., using the membrane filter technique (Standard Methods, 14th Ed., 1975).

Pathogenic Naegleria amoebae were isolated only from samples obtained from the swimming pool. Both 50 gallon water samples were positive, as were three of the four sediment samples and one of the five 20 ml water samples. The chemical and bacteriological findings were within acceptable ranges. The apparent absence of pathogenic Naegleria in the two feeder ponds may simply reflect the limited number of samples obtained.

Texas

In August, 1977, about two weeks after the Georgia case, PAM was reported in a teenage female who had been swimming in a polluted Texas lake immediately prior to onset of illness. The Texas State Department of Health submitted samples from the suspected exposure site to ERC for isolation attempts. The specimens included five 200 ml water samples and 1 liter of sediment. Two plate cultures of amoebae isolated by the Texas State Department of Health were submitted at the same time.

The water specimens were processed by the membrane filter method and the sediment processed as described earlier. All were negative for pathogenic Naegleria. The two plate cultures submitted proved to be Naegleria gruberi,

a nonpathogen. Specimens were in transit at ambient temperatures for 72 hours, which may account for the negative findings.

South Carolina

In September, 1977, a case of PAM was reported from Charleston, S.C. The patient was a young boy who had a recent history of swimming in a large, man-made lake in the Charleston area. The South Carolina Department of Health submitted two gallons of water and five 200 ml sediment samples that were collected from the suspect lake.

Only one of the sediment samples yielded pathogenic Naegleria. No isolates were obtained from the water. This reinforces the axiom that negative results have little validity. If the sediment samples had not been submitted the only evidence incriminating the suspect exposure site would have been circumstantial.

Florida

On July 5, 1978, a 14-year-old boy with a history of swimming was tentatively diagnosed as having PAM based on the observation of amoebae in the cerebral spinal fluid (CSF). A portion of this CSF was delivered to ERC at 10:00 A.M. Aliquots were inoculated onto an IA plate with E. aerogenes and into CSYECM and incubated at 37°C. At 2:00 P.M. amoebae from the CSYECM culture were examined for flagellate transformation and by the IFA test. Both were positive for pathogenic Naegleria. Thus, the diagnosis was confirmed within four hours of receipt of the specimen. A field team went to the lake suspected of being the exposure site. Ten 200 ml sediment samples were collected from various sites on the lake. Two liters of water were obtained for MPN determinations of Naegleria. One liter was taken where the boy had been seen swimming and the other across the lake at a beach where most of the swimming activity usually occurs.

Even though the MPN index was <2 pathogenic Naegleria per liter at the site where the boy had been swimming, three of the four sediment samples from the same site were positive. Conversely, the MPN index for the beach across the lake was two pathogenic Naegleria per liter but both sediment samples were negative. Representative organisms from each positive sample and from the CSF were confirmed by mouse intranasal instillation.

California

In May, 1978, a case of PAM was diagnosed in a nine-year-old California girl. One week before onset she had bathed in a hot springs near San Bernandino. This same hot springs had been implicated in a 1971 case of PAM.

On July 12, 1978, and August 22, 1979, specimens were received from the California Department of Health for isolation of pathogenic Naegleria. A subculture of the amoebae reportedly isolated from her initial CSF specimen was requested but has not been received.

From the first set of specimens obtained from the hot springs, all four

sediment samples were negative for pathogenic Naegleria. From the second set obtained from a stream near the springs, no pathogens were isolated but seropositive nonpathogenic Naegleria were isolated from two of the six sediment samples. Once again, these negative samples do not preclude the possibility that pathogens are present in the springs or adjoining streams. Additional testing should be done.

South Carolina

In August, 1978, a case of PAM was reported from Charleston, S.C., for the second straight year. The possible exposure sites consisted of a drainage ditch and the same man-made lake that had been implicated the previous year.

Both water and sediment samples submitted from the ditch failed to yield pathogenic Naegleria. However, seven of the 12 sediment samples representing all sites sampled from the man-made lake were positive for pathogenic Naegleria. These data reinforced the validity of the isolate obtained from this same lake the year before.

Florida

In late August, 1978, a nine-year-old white male from Bakers County, Florida, succumbed to a fatal PAM infection. A week after his death, ERC staff members went to Baker County and obtained water and sediment samples from the four suspect swimming sites. Samples obtained from each site included a 50 gallon water sample and four 100 ml sediment samples obtained from different areas. A MPN test was done on one site at each swimming area. None of the 50 gallon water samples yielded pathogenic Naegleria, which was surprising in view of the fact that two of the four sites showed two amoebae per liter. Only three of the 16 sediment samples yielded pathogenic Naegleria These were all from Clay Pond which had shown two pathogens per liter in the MPN test and which was the swimming site nearest to the infected boy's home. Water temperature in both of the negative sites was 26°C, whereas water temperatures in the two positive sites were 31°C and 33°C.

Additional Supportive Activities

Comparable surveillance studies for pathogenic Naegleria had been initiated in Virginia under the direction of Richard Duma, M.D. He and members of his staff therefore spent several days at ERC observing techniques. As a control for both laboratories specimens were shared.

In May, 1978, environmental specimens were received from Dr. Duma. At that time, pathogenic Naegleria had not been isolated from any of their environmental samples. Samples processed at ERC yielded one strain of seropositive nonpathogenic Naegleria but no pathogenic Naegleria were obtained. These data would appear to support their negative findings.

On July 8, four 200 ml sediment samples were collected from different sites at a known positive lake in Florida. Each sample was well mixed, divided into two equal samples and placed in resealable plastic bags. These

were incubated at 43°C. On the afternoon of July 10, 1978, one of each set was packed and shipped via United Parcel Service to Dr. Duma's laboratory and the companion set placed at room temperature. Upon notification that the samples had arrived and processing was about to be initiated, the four companion samples held at room temperature were processed. Results were identical in both laboratories; one sample was negative and three were positive. These data confirm the reliability of techniques employed.

When ERC personnel assisted in the epidemiological investigation of the fatal PAM case which occurred in Hephzibah, Georgia in July, 1977, Georgia State officials requested that a survey be taken for pathogenic Naegleria in Georgia lakes. With EPA approval and additional funding, two ERC staff members, utilizing ERC's Mobile Laboratory, surveyed lakes in Georgia from 6 to 18 August, 1978.

Specimens were collected from 20 lakes representing 16 counties. Two sites were sampled on all but one lake. Specimens obtained from each lake included two 50 gallon water samples, six or eight 100 ml sediment samples, and two water samples for the on-site MPN pathogenic Naegleria determination. Comparable water samples were collected for chemical and bacteriological studies. The former were done using a Hach DR-EL/2 kit (Hach Chemical Co., Loveland, Colorado). The latter analyses were performed by the Chatham County Department of Health, Chatham County, Georgia, using the multiple tube method to enumerate total and fecal coliforms and fecal streptococci. Water samples were delivered to the laboratory within six hours. Results are included in this report.

Samples for pathogenic Naegleria isolations were incubated at 43°C until shipped to the base laboratory, where they were again incubated at 43°C until processed. These incubation histories are shown on Table 21. Samples were processed as previously described and amoebae from at least one plaque from each positive sample were tested for mouse pathogenicity by intranasal instillation. These were all confirmed.

Seven of the 20 lakes (35%) tested yielded pathogenic Naegleria from at least one of the samples tested. All seven positive lakes were located south of Atlanta, or below the so-called Fall Line. Thirteen of the 20 lakes tested were south of Atlanta giving a 53.8% (7/13) positivity for lakes in that geographic area (see Table 22).

Chemistry data varied little from lake to lake and were within the range seen in Florida's freshwater lakes. No real differences could be seen between negative and positive lakes (see Tables A-10 and A-11, Appendix A).

Bacteriological findings are shown in Table 23 according to lake and site sampled. Bacterial densities varied extremely among the lakes and between sites on the same lakes. These data are summarized on Table 24 according to the geographic location of the lake in relation to Atlanta and the presence or absence of pathogenic Naegleria. In lakes located south of Atlanta, there does appear to be a trend toward higher bacterial counts in the positive lakes based on the median. Using the F and t tests designed for data showing extensive variations, the differences were not significant at

TABLE 21. INCUBATION HISTORIES OF SAMPLES FROM SELECTED GEORGIA LAKES FOR PATHOGENIC *NAEGLERIA* ISOLATION

Lake	Number of hours held at 43°C in mobile laboratory	Number of hours held at Various temperatures in transit*	Number of hours held at 43°C in base laboratory
Griffin	72	30	48
Cypress	72	30	48
Carrol	48	30	48
Olmstead	48	30	48
Lester	72	30	12
Deltina	72	30	12
Fort Yargo	48	30	12
Yonah	48	30	12
Sky	24	30	12
Dockery	24	30	12
Blackburn	120	0**	96
Indian	120	0	96
Spivey	96	0	96
Ransby	96	0	96
Robin	72	0	96
Concharty	72	0	96
Tobesofkee	48	0	120
Houston	48	0	120
Blackshear	24	0	120
Long	24	0	120

* Temperature not known but undoubtedly wide variations.
** Returned to base laboratory in mobile laboratory.

TABLE 22. LOCATION OF LAKES, WATER TEMPERATURE, AND RESULTS OF PATHOGENIC *NAEGLERIA* ISOLATION ATTEMPTS

Lake	Geographic location with respect to Atlanta		Lake water temperature °C	*Naegleria* MPN* index per liter of water	Number positive/ number tested	
	North	South			Water samples	Sediment samples
Griffin		X	29.0	5, 5	1/4	0/6
Cypress		X	29.5	5,<2	0/4	0/6
Carrol		X	30.0	5,<2	0/2	3/8
Olmstead		X	32.0	<2,<2	0/2	1/8
Lester	X		28.5	<2,<2	0/2	0/8
Deltina	X		31.0	<2,<2	0/2	0/8
Fort Yargo	X		27.0	<2,<2	0/2	0/8
Yonah	X		25.0	<2,<2	0/2	0/8
Sky	X		24.0	<2,<2	0/2	0/8
Dockery	X		21.0	<2, ND	0/1	0/4
Blackburn	X		24.5	<2,<2	0/2	0/8
Indian		X	30.5	<2,<2	0/2	0/8
Spivey		X	27.5	<2, 2	0/2	1/8
Ransby		X	33.0	<2,<2	0/2	0/8
Robin		X	28.5	<2,<2	0/2	1/8
Concharty		X	29.0	<2,<2	0/2	0/8
Tobesofkee		X	29.0	<2,<2	0/2	0/8
Houston		X	33.5	<2,<2	0/2	0/8
Blackshear		X	31.0	<2,<2	0/2**	0/8
Long		X	34.0	5,<2	0/2	0/8

* Two sites tested except when indicated ND (not done).
** Seropositive nonpathogenic *Naegleria* isolated.

TABLE 23. BACTERIOLOGICAL DATA AND THE PRESENCE OR ABSENCE OF PATHOGENIC NAEGLERIA IN GEORGIA LAKES SAMPLED

Lake	Site	Pathogenic Naegleria: Water neg.	Water pos.	Sediment neg.	Sediment pos.	Bacterial results: Coliform MPN	Fecal coliform	Fecal strep.
Griffin	1		+	-		9,200	79	260
	2	-		-		16,000	1,720	>24,000
Cypress	1	-		-		141	17	5,420
	2	-		-		>24,000	9,200	542
Carrol	1	-			+	11	2	221
	2	-			+	23	2	33
Olmstead	1	-			+	3,480	920	79
	2	-		-		3,480	460	221
Lester	1	-		-		542	<2	33
	2	-		-		211	14	46
Deltina	1	-		-		5,400	920	240
	2	-		-		172	2	175
Fort Yargo	1	-		-		3,480	45	49
	2	-		-		2,400	49	1,600
Yonah	1	-		-		1,600	172	109
	2	-		-		2,400	109	49
Sky	1	-		-		BIT*	BIT	BIT
	2	-		-		2,400	109	700
Dockery	1	-		-		2,400	543	16,000
	2	ND**		ND		3,480	120	>24,000
Blackburn	1	-		-		1,720	130	49
	2	-		-		9,200	79	70
Indian	1	-		-		3,480	17	33
	2	-		-		348	348	17
Spivey	1	-		-		33	<2	<2
	2	-			+	BIT	BIT	BIT
Ransby	1	-		-		920	8	348
	2	-		-		79	13	22
Robin	1	-		-		348	49	240
	2	-			+	109	5	17
Concharty	1	-		-		70	8	<2
	2	-		-		46	7	<2
Tobesofkee	1	-		-		221	49	17
	2	-		-		221	33	49
Houston	1	-		-		460	130	7
	2	-		-		790	23	33
Blackshear	1	-		-		22	5	49
	2	-†		-		23	7	5
Long	1	-		-		1,410	46	221
	2		+	-		BIT	BIT	BIT

* BIT - Broken in transit.

** ND - Not done.

† Seropositive nonpathogenic Naegleria isolated.

the 5% level. With 2.201 being significant for the 5% level, the t value for total coliforms was 1.91, for fecal coliforms, 1.305 and for fecal streptococci, 1.28. Although significance cannot be established, there does appear to be a trend toward higher bacterial densities in the positive lakes as opposed to the negative ones. If more extensive testing were done on selected lakes, significance may be demonstrated. If so, perhaps more frequent bacterial analyses of public swimming areas during July and August and strict adherence to swimming restrictions in case of increased bacterial counts, could represent a first line control against human PAM infections.

TABLE 24. BACTERIAL DENSITY RANGES AS RELATED TO THE GEOGRAPHICAL LOCATION OF THE LAKE WITH RESPECT TO ATLANTA AND THE PRESENCE OR ABSENCE OF PATHOGENIC *NAEGLERIA*

Lake location in relation to Atlanta	Pathogenic *Naegleria*	Total coliforms		Fecal coliforms		Fecal streptococci	
		Range	Median	Range	Median	Range	Median
North (7)*	Absent	172- 9,200	2,400	<2- 920	109	33->24,000	109
South (7)	Present	11-24,000	531	<2-9,200	47.5	<2->24,000	221
South (6)	Absent	22- 3,480	221	5- 348	15	<2 348	19.5

*Number of lakes sampled shown in parentheses.

Ample data show a positive relationship between increased water temperatures and the presence of pathogenic *Naegleria*. This was evidenced also in this study. The average water temperature of the positive lakes was 30.1°C, whereas that of the negative lakes was 28.2°C. However, exceptions were noted. The water temperature in one negative lake was 33.5°C and in one positive lake, only 27.5°C Other factors, perhaps even sampling sites and frequency, play a role in the isolation of pathogenic *Naegleria* amoebae from any given lake, particularly from those lakes in which the water temperature is ≥28°C. All study lakes situated north of Atlanta were negative and had an average water temperature of 25.9°C. Conversely, 53.8% of the study lakes located south of Atlanta were positive and the average water temperature was 30.5°C. This would appear to indicate that water temperature played an important role in the negative status of lakes located north of Atlanta.

The one enigma encountered in this study was the failure to demonstrate pathogenic *Naegleria* in large water samples from sites where a positive MPN test was found. Previous experiences in Florida have resulted in negative MPN tests and positive large water samples. One possible explanation for this may be the on-site inoculation of the MPN concentrate, as opposed to the long-term incubation of the large water sample sand column containing the concentrates. The latter were incubated under crowded conditions, which perhaps resulted in temperature fluctuations due to poor circulation. As has

been shown in laboratory situations, vacillating temperatures are much more deleterious to these organisms than is a constant temperature, even room temperature. Temperature fluctuations may help to account for the poor yield from the large water samples.

Laboratory studies had shown that a 96-hour incubation period at 43°C increased the probability of isolating pathogenic Naegleria from sediment samples. It was assumed that the same treatment should be beneficial for large water sand column concentrates, but the procedure had not been field tested. In view of the poor yield (one positive sample) from large water samples and the relatively good yield (six positive samples) from the MPN water samples, one questions whether or not predators or toxic substances were also concentrated and brought into close proximity with the amoebae. The long enrichment period could have enhanced predation and/or deleterious toxic effects due to the concentration or proximity. In the case of sediments, a more natural state exists and incubation apparently enhances multiplication of the pathogens.

This survey has confirmed data accrued in Florida, indicating that pathogenic Naegleria amoebae are widely distributed in freshwater lakes in which the water temperature is elevated over a period of time. The occurrence of cases in Virginia precludes limiting the geographical distribution to the more southern United States.

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APPENDIX A

TABLES

TABLE A-1. SAMPLE DATA AND RESULTS OF TESTS ON THERMALLY ENRICHED LAKES (January, 1976 - March, 1978)

		Water sample			Sediment sample		
Date	Water temp. °C	Volume (liters)	Depth (meters)	Number positive/ number tested	Volume (ml)	Depth (meters)	Number positive/ number tested
			Lake Monroe				
01-27-76	25.0	757.1	<0.3	0/1			
		378.5	<0.3	0/1			
02-03-76	23.0	189.2	<0.3	0/1	100	<0.3	0/1
		0.125	<0.3	0/2			
02-10-76	16.0	189.2	<0.3	0/1	100	<0.3	0/2
		0.15	<0.3	0/1			
		0.10	<0.3	0/1			
02-24-76	26.0	189.2	<0.3	0/1	100	<0.3	1/3
		0.15	<0.3	0/1			
		0.10	<0.3	0/1			
03-08-76	32.0	0.60	<0.3	1/2	100	<0.3	0/2
03-30-76	35.0	378.5	<0.3	1/1	100	<0.3	0/3
05-04-76	25.0	0.1	<0.3	0/1	100	<0.3	0/1
		0.2	<0.3	0/15			
05-10-76	30.0	0.2	<0.3	0/1	100	<0.3	0/1
		0.16	<0.3	0/7			
06-07-76	34.0	0.10	<0.3	0/2			
09-27-76	36.0	2.0	<0.3	0/1			
10-25-76	26.5	189.2	<0.3	1/1			
11-04-76	28.0	189.2	<0.3	0/1			
11-20-76	31.0	189.2	<0.3	1/1			
12-27-76	23.0	189.2	<0.3	1/1			
01-31-77	21.5	189.2	<0.3	0/1	200	<0.3	1/1
03-01-77	31.0	189.2	<0.3	0/1	100	<0.3	0/1
03-28-77	26.0	189.2	<0.3	1/1	200	<0.3	0/1
04-14-77	23.0	189.2	<0.3	1/1	200	<0.3	0/1
05-26-77	37.0	189.2	<0.3	0/1	200	<0.3	0/1
06-23-77	38.0	189.2	<0.3	0/1	100	<0.3	0/1
07-27-77	41.0	189.2	<0.3	0/1	200	<0.3	0/1

(continued)

TABLE A-1 (continued)

Date	Water temp.°C	Water sample Volume (liters)	Depth (meters)	Number positive/ number tested	Sediment sample Volume (ml)	Depth (meters)	Number positive/ number tested
			Lake Sanford				
02-20-78	17.0				200	1.5	0/6
	17.0				200	2.4	0/6
	18.0				200	4.3	0/9

TABLE A-2. SAMPLE DATA AND RESULTS OF TESTS ON LAKE CONWAY
(January, 1976 - March, 1978)

		Water sample			Sediment sample		
Date	Water temp. °C	Volume (liters)	Depth (meters)	Number positive/ number tested	Volume (ml)	Depth (meters)	Number positive/ number tested
02-03-76	19.0	378.54	<0.3	0/1	100	<0.3	0/1
		0.002	<0.3	0/1	100	<0.3	0/1
03-08-76	26.0	.0015	<0.3	0/1	100	<0.2	0/1
		283.9	<0.3	0/1			
03-16-76	24.0	189.2	<0.3	0/1	200	<0.3	0/1
03-23-76	22.0	189.2	<0.3	0/1	300	<0.3	0/1
04-06-76	24.0	189.2	<0.3	0/1	300	<0.3	0/1
04-19-76	26.0	189.2	<0.3	0/1	200	<0.3	0/1
05-17-76	31.0	189.2	<0.3	0/1	100	<0.3	0/1
06-14-76	29.0	189.2	<0.3	0/1	100	<0.3	0/1
06-21-76	31.0	189.2	<0.3	0/1	100	<0.3	0/1
07-12-76	30.0	189.2	<0.3	0/1	100	<0.3	0/1
07-19-76	31.0	189.2	<0.3	0/1	200	<0.3	0/1
07-26-76	32.0	94.6	<0.3	1/1	100	<0.3	0/1
08-02-76	33.0	0.2	<0.3	1/5	100	<0.3	1/1
		0.1	<0.3	1/5			
		0.05	<0.3	1/5			
		0.025	<0.3	1/5			
08-09-76	34.0	94.6	<0.3	1/1	100	<0.3	1/1
09-28-76	29.0	2.0	<0.3	0/1	100	<0.3	1/1
10-18-76	26.0	2.0	<0.3	0/1	100	<0.3	1/1
10-25-76	23.5	2.0	<0.3	0/1	100	<0.3	0/1
11-02-76	25.0	189.2	<0.3	0/1	200	<0.3	0/1
11-08-76	20.5	189.2	<0.3	0/1	200	<0.3	0/3
11-30-76	18.0				200	<0.3	0/3
12-02-76	16.0				100	2.4	1/10
					100	2.1	0/2
12-13-76	15.5				100	6.1	1/2
					100	7.6	2/2
					100	2.1	0/4
					100	2.4	1/4
01-10-77	16.0				200	7.6	2/2
					200	2.1	0/6
					200	2.4	0/4
01-24-77	12.0				200	7.3	4/4
					200	2.4	0/5
02-10-77	14.0				100	5.5	5/6
					100	8.2	2/3
					100	5.5	1/2
03-03-77	17.5				100	2.4	0/4
					100	6.7	4/4
					100	9.7	1/4

(continued)

TABLE A-2 (continued

Date	Water temp. °C	Water sample Volume (liters)	Depth (meters)	Number positive/ number tested	Sediment sample Volume (ml)	Depth (meters)	Number positive/ number tested
03-14-77	22.0				100	2.4	0/4
					100	7.3	4/4
					100	8.2	2/3
					100	8.5	3/3
03-24-77	26.0	189.2	<0.3	0/1	100	<0.3	0/2
03-28-77	26.0				100	<0.3	0/1
03-31-77	23.5	189.2	<0.3	0/1	100	<0.3	0/1
04-07-77	25.0	189.2	<0.3	0/1	200	<0.3	0/1
04-11-77	23.5				100	2.3	0/3
					100	2.4	0/6
					100	6.4	4/6
					100	7.3	2/3
04-14-77	24.0				200	<0.3	0/1
04-25-77	26.0	189.2	<0.3	0/1	200	<0.3	0/1
05-02-77	26.0	189.2	<0.3	0/1	200	<0.3	0/1
05-12-77	25.0	189.2	<0.3	0/1	200	<0.3	0/1
05-16-77	26.0				100	2.4	0/4
					100	6.4	4/4
					100	7.3	4/4
					100	8.2	3/4
05-19-77	28.0	189.2	<0.3	0/1	200	<0.3	0/1
05-26-77	30.0	189.2	<0.3	0/1	200	<0.3	0/1
06-01-77	30.5	189.2	<0.3	0/1	350	<0.3	0/1
06-08-77	29.0	189.2	<0.3	0/1	300	<0.3	0/1
06-13-77	30.5	189.2	<0.3	0/1	300	<0.3	0/1
06-16-77	31.0	189.2	<0.3	0/1	300	<0.3	0/1
06-20-77	29.5				150	2.1	2/4
					150	4.9	1/4
					150	6.4	5/8
					150	8.2	0/4
06-23-77	31.5	189.2	<0.3	0/1	300	<0.3	0/1
07-11-77	32.5	189.2	<0.3	0/1	300	<0.3	0/1
07-14-77	32.0	189.2	<0.3	0/1	300	<0.3	0/1
07-21-77	32.0	189.2	<0.3	0/1	200	<0.3	0/1
07-22-77	30.0	1.0	<0.3	0/1			
		1.0	0.6	0/1			
		1.0	2.1	0/1			
07-28-77	32.0	189.2	<0.3	0/1	200	<0.3	0/1
08-29-77	29.0	6.0	2.4	1/1	200	<0.3	0/1
12-08-77	19.0				200	2.4	0/4
	19.0				200	2.1	0/4
	19.5				200	6.1	4/4
	19.5				200	9.4	0/4
	19.5				200	2.4	0/4

(continued)

TABLE A-2 (continued)

Date	Water temp.°C	Water sample Volume (liters)	Depth (meters)	Number positive/ number tested	Sediment sample Volume (ml)	Depth (meters)	Number positive/ number tested
01-05-78	15.5				200	5.2	2/3
	16.0				200	6.7	0/3
	15.5				200	2.3	0/3
	16.0				200	5.5	2/3
	16.0				200	8.5	0/3
	16.0				200	9.4	1/3
01-23-78	15.2				400	7.9	4/4
02-02-78	14.5				200	2.1	0/3
	14.0				200	6.1	3/3
	14.0				200	6.4	3/3
	14.0				200	7.9	4/6
02-13-78	14.5				200	2.1	0/3
	14.0				200	6.4	3/3
	14.0				200	7.9	1/3
	16.5				200	2.3	0/3
	15.0				200	6.1	3/3
	14.5				200	8.2	1/3
	16.5				200	2.3	0/3
	15.0				200	6.1	3/3
	14.5				200	8.2	3/3
03-16-78	18.0				200	6.7	14/18

TABLE A-3. SAMPLE DATA AND RESULTS OF TESTS ON LAKE HOURGLASS
(January, 1976 - March, 1978)

		Water sample			Sediment sample		
Date	Water temp. °C	Volume (liters)	Depth (meters)	Number positive/ number tested	Volume (ml)	Depth (meters)	Number positive/ number tested
01-13-76	17.0	1,135.6	<0.3	0/1			
		0.5	<0.3	0/1			
03-16-76	25.0				200	<0.3	0/1
03-23-76	24.0				300	<0.3	0/1
04-19-76	26.0	189.2	<0.3	0/1	200	<0.3	0/1
05-17-76	28.5				100	<0.3	0/2
06-14-76	29.5	94.6	<0.3	1/1	100	<0.3	1/1
06-21-76	30.0	94.6	<0.3	1/1	100	<0.3	0/1
06-28-76	30.0	0.1	<0.3	4/5	100	<0.3	2/2
		0.05	<0.3	0/1			
		94.6	<0.3	1/1			
07-12-76	33.0	0.2	<0.3	5/5			
		0.1	<0.3	4/5			
		0.05	<0.3	5/5			
		0.025	<0.3	5/5			
10-11-76	26.0	2.0	<0.3	1/1	100	<0.3	0/1
10-18-76	26.0	2.0	<0.3	0/1	100	<0.3	0/1
11-02-76	20.0	189.2	<0.3	0/1	200	<0.3	0/1
11-08-76	19.0	189.2	<0.3	0/1	200	<0.3	0/3
11-18-76	21.0				200	4.9	0/8
					200	2.4	0/4
					200	2.1	0/2
12-09-76	15.5				100	2.7	0/2
					100	3.0	0/2
					100	3.6	0/3
					100	3.3	0/5
01-03-77	16.0				150	1.2	0/2
					150	1.8	0/2
					150	2.1	0/4
					150	2.4	0/4
01-17-77	14.5				200	1.2	0/1
					200	3.9	0/2
					200	3.6	0/4
				(cattail roots)	200	Shoreline	0/1
02-03-77	14.0				100	3.0	0/10
02-24-77	18.0				100	3.0	0/12
03-10-77	21.0				100	2.7	0/3
					100	3.0	0/3
					100	2.7	1/3
					100	2.4	0/3
04-04-77	27.5				400	3.3	0/4
					1,000	3.0	0/9*

(continued)

TABLE A-3 (continued)

Date	Water temp.°C	Water sample Volume (liters)	Depth (meters)	Number positive/ number tested	Sediment sample Volume (ml)	Depth (meters)	Number positive/ number tested
04-07-77	25.0	189.2	<0.3	0/1	400	<0.3	0/2
04-14-77	24.0				200	<0.3	0/1
04-25-77	26.0	189.2	<0.3	0/1	200	<0.3	0/1
05-09-77	29.0	5.0	2.7	0/1	1,800	2.7	0/18†
05-12-77	26.0	189.2	<0.3	0/1	200	<0.3	0/1
05-19-77	29.0	189.2	<0.3	0/1	200	<0.3	0/1
05-26-77	31.0	189.2	<0.3	0/1	200	<0.3	0/1
06-01-77	31.5	189.2	<0.3	0/1	375	<0.3	0/1
06-06-77	28.5	1.0	3.0	0/1	400	3.0	0/4
		1.0	3.3	0/1	400	3.3	0/4
		1.0	2.7	0/2	400	2.7	0/8*
06-08-77	--**	1.0	<0.3	0/1	300	<0.3	0/1
06-13-77	31.0	189.2	<0.3	0/1	300	<0.3	0/1
06-16-77	32.0	189.2	<0.3	0/1	300	<0.3	0/1
06-23-77	31.0	189.2	<0.3	0/1	300	<0.3	0/1
07-11-77	33.0	189.2	<0.3	0/1	300	<0.3	0/1
07-14-77	32.0	189.2	<0.3	0/1	300	<0.3	1/1
07-18-77	30.5	1.0	3.0	1/1	400	3.0	4/4
		1.0	2.7	1/2	400	2.7	8/8*
		1.0	<0.3	1/1	400	<0.3	0/2
07-21-77	32.0	189.2	<0.3	0/1	200	<0.3	0/1
07-28-77	33.5	189.2	<0.3	0/1	200	<0.3	0/1
02-02-78	15.0				200	3.0	0/9
					200	3.3	0/3
					200	1.8	0/3

* Taken at two different sites.
† Taken at six sites.
** Not recorded.

TABLE A-4. SAMPLE DATA AND RESULTS OF TESTS ON LAKE BALDWIN
(January, 1976 - March, 1978)

		Water sample			Sediment sample		
Date	Water temp.°C	Volume (liters)	Depth (meters)	Number positive/ number tested	Volume (ml)	Depth (meters)	Number positive/ number tested
07-26-76	31.0	94.6	<0.3	1/1	100	<0.3	1/1
08-02-76	32.0	0.2	<0.3	5/5			
		0.1	<0.3	5/5			
		0.05	<0.3	5/5			
		0.025	<0.3	5/5			
08-09-76	31.0	94.6	<0.3	1/1	100	<0.3	1/1
09-27-76	32.5	2.0	<0.3	1/1	100	<0.3	0/1
10-18-76	24.5	2.0	<0.3	1/1	100	<0.3	1/1
10-25-76	25.0	2.0	<0.3	0/1	100	<0.3	0/1
		189.2	<0.3	0/1	200	<0.3	0/1
11-08-76	18.0	189.2	<0.3	0/1	200	<0.3	0/3
11-15-76	21.0				200	1.8	0/2
					200	1.2	0/2
					200	1.8	0/2
					200	2.1	5/8
11-29-76	21.0				200	<0.3	0/3
12-06-76	17.0				100	2.1	5/6
					100	4.6	0/1
					100	1.2	0/2
12-16-76	19.0				100	2.4	3/10
01-13-77	14.5				200	2.1	1/4
					200	5.5	3/4
					200	2.1	0/2
					200	1.2	0/2
01-27-77	12.0				200	5.5	2/6
					200	2.4	0/2
					200	2.1	1/4
02-14-77	16.0				100	5.2	2/6
					100	2.1	2/6
03-07-77	21.5				100	2.1	2/4
					100	5.5	4/8
03-21-77	26.0				100	2.1	0/4
					100	5.5	0/4
					100	5.8	2/4
					100	<0.3	0/4
04-07-77	26.0	189.2	<0.3	0/1	200	<0.3	0/1
04-14-77	24.0				200	<0.3	0/1
04-18-77	26.0	1.0	2.3	0/1	100	2.3	1/3
		3.0	5.5	0/1	100	5.5	3/9
04-21-77	26.0	189.2	<0.3	0/1	200	<0.3	0/1
05-05-77	26.0	189.2	<0.3	0/1	200	<0.3	0/1
05-12-77	25.0	189.2	<0.3	0/1	200	<0.3	0/1

(continued)

TABLE A-4 (continued)

Date	Water temp.°C	Water sample Volume (liters)	Depth (meters)	Number positive/ number tested	Sediment sample Volume (ml)	Depth (meters)	Number positive/ number tested
05-26-77	27.0	1.0	3.0	0/1	100	2.4	0/4
		1.0	3.0	0/1	100	4.6	1/4
		1.0	3.0	0/1	100	5.8	2/4
		1.0	3.0	0/1	100	5.5	1/4
		1.0	2.1	0/1	100	2.3	0/4
06-01-77	29.0	189.2	<0.3	0/1	350	<0.3	0/1
06-08-77	27.0	189.2	<0.3	0/1	300	<0.3	0/1
06-13-77	29.0	189.2	<0.3	0/1	300	<0.3	0/1
06-15-77	29.0	189.2	<0.3	0/1	300	<0.3	0/1
06-23-77	29.5	189.2	<0.3	0/1	300	<0.3	0/1
07-11-77	31.0	189.2	<0.3	0/1	300	<0.3	1/1
07-14-77	31.0	189.2	<0.3	0/1	300	<0.3	1/1
07-21-77	30.5	189.2	<0.3	0/1	200	<0.3	1/1
07-28-77	32.5	189.2	<0.3	0/1	200	<0.3	0/1
08-01-77	30.5	1.0	<0.3	1/1			
		1.0	0.6	1/1			
		1.0	2.4	1/1			
08-26-77	30.0	2.0	<0.3	1/1	200	<0.3	1/1
		2.0	0.6	1/1			
		2.0	2.4	1/1			
09-26-77	32.0	189.2	<0.3	0/1	200	<0.3	0/2
11-14-77	20.0				200	<0.3	0/4
	19.0				200	2.1	0/2
	19.0				200	5.8	0/4
	19.0				200	5.3	3/4
	19.0				200	6.1	2/4
01-16-78	13.2				200	5.2	4/6
	13.5				200	2.4	0/3
	15.2				200	5.5	2/6

TABLE A-5. SAMPLE DATA AND RESULTS OF TESTS ON LAKE SPIER
(January, 1976 - March, 1978)

		Water sample			Sediment sample		
Date	Water temp. °C	Volume (liters)	Depth (meters)	Number positive/ number tested	Volume (ml)	Depth (meters)	Number positive/ number tested
01-20-76	14.0	378.5	<0.3	0/3			
07-26-76	32.0	94.6	<0.3	0/1	100	<0.3	0/1
08-02-76	31.5	189.2	<0.3	0/1	100	<0.3	0/1
08-09-76	32.0	189.2	<0.3	0/1	100	<0.3	0/1
08-16-76	34.5	189.2	<0.3	0/1	100	<0.3	0/1
09-02-76	31.0	189.2	<0.3	0/1	200	<0.3	0/1
		113.6	<0.3	0/1			
09-27-76	33.0	189.2	<0.3	0/1	100	<0.3	0/1
10-11-76	26.0	189.2	<0.3	0/1	100	<0.3	0/1
10-18-76	25.0	189.2	<0.3	0/1	100	<0.3	0/1
10-25-76	26.5	189.2	<0.3	0/1	100	<0.3	0/1
04-14-77	24.0				200	<0.3	0/1
04-21-77	26.0	189.2	<0.3	0/1	200	<0.3	0/1
04-25-77	24.0	189.2	<0.3	0/1	200	<0.3	0/1
05-05-77	26.0	189.2	<0.3	0/1	200	<0.3	0/1
05-12-77	26.0	189.2	<0.3	1/1*	200	<0.3	0/1
05-19-77	26.5	189.2	<0.3	0/1	200	<0.3	0/1
05-26-77	29.0	189.2	<0.3	0/1	200	<0.3	0/1
06-01-77	29.0	189.2	<0.3	0/1	200	<0.3	1/1*
06-08-77	27.5				300	<0.3	0/1
06-16-77	29.5	189.2	<0.3	0/1	300	<0.3	0/3
06-23-77	30.0	189.2	<0.3	0/1	300	<0.3	1/2†
07-11-77	31.0	189.2	<0.3	1/1	300	<0.3	0/2
07-14-77	31.0	189.2	<0.3	0/1	300	<0.3	1/2
07-21-77	30.0	189.2	<0.3	1/1	200	<0.3	0/1
07-28-77	31.0	189.2	<0.3	0/1	200	<0.3	0/1
08-01-77	31.0	1.0	<0.3	1/1			
		1.0	0.6	0/1			

* Only one of 20 plates yielded a pathogenic Naegleria plaque.
† Only one of 40 plates were positive with a single plaque.

TABLE A-6. SAMPLE DATA AND RESULTS FROM ORANGE COUNTY LAKES SAMPLED INFREQUENTLY (January, 1976 - March, 1978)

Lake	Date sampled	Water temp. °C	Water sample		Sediment sample	
			Volume (liters)	Number positive/ number tested	Volume (ml)	Number positive/ number tested
Killarney	02-10-76	18.0	.25	0/1	100	0/1
	08-16-76	32.0	94.6	1/1	100	1/1
Virginia	02-10-76	20.5	.25	0/1	100	0/1
	08-23-76	30.5	94.6	1/1	100	0/1
Bell	02-10-76	21.0	.25	0/1	100	0/1
	10-11-76	25.5	2.0	1/1	100	0/1
Osceola	02-10-76	18.0	.25	0/1	100	0/1
	10-07-76	28.0	2.0	1/1	100	0/1
Fairview	08-23-76	30.5	94.6	1/1	100	1/1
Mary Jane	08-23-76	31.5	94.6	1/1	100	1/1
Clear	09-02-77	27.5	189.2	1/1	200	0/1
Holden	09-02-77	28.0	189.2	1/1	200	0/1
Mann	09-02-77	27.5	189.2	0/1	200	0/1
	09-22-77	30.5	189.2	1/2	200	1/2
	09-29-77	30.0	189.2	1/1	200	1/1
Apopka	09-22-77	29.0	189.2	1/1	200	0/1
Mary Gem	09-22-77	34.5	189.2	1/1	200	0/1
Telfer	09-26-77	30.0	189.2	0/1	200	0/2
	10-17-77	21.0	189.2	0/1	200	0/3
Lucern	09-26-77	32.0	189.2	1/1	200	1/1
Turkey	10-06-77	30.0	189.2	0/1	200	0/1
Bear Gully	10-10-77	27.0	189.2	1/1	200	0/1
Irma	10-10-77	28.0	189.2	0/1	200	0/1

TABLE A-7. RESULTS OF SURVEY FOR PATHOGENIC NAEGLERIA IN LAKE WATER AND SEDIMENT SAMPLES FROM LAKES OUTSIDE OF ORANGE COUNTY (January, 1976 - March, 1978)

				Samples			
				Water		Sediment	
County	Lake	Date	Water temp. °C	Size (L)	Results	Size (ml)	Results
Collier	Trafford	10-03-76	27.5	189.2	+	100	-
Gadsden	Seminole	09-13-76	28.0			200	-
	Talquin	06-29-77	30.0	2	-	300	-
Hernando	Large Camp-A-Wyle	08-30-76	32.0	2	+	100	+
	Small Camp-A-Wyle	08-30-76	32.0	2	-	100	+
Highlands	Placid	09-09-76	32.0	189.2	-	200	-
Hills-borough	Thonotosassa	08-30-76	33.0	2	-	100	-
		10-13-77	24.0	189.2	-	200	-
	Brotherhood Union	09-20-76	30.0	189.2	+	200	-
	Magdaline	09-20-76	33.0	189.2	+	200	+
	Egypt	09-20-76	32.5	189.2	-	200	-
		10-13-77	26.0	189.2	+	200	-
	Hiawatha	10-07-76	28.0	189.2	+	100	-
	Keystone	10-07-76	28.5	189.2	-	100	-
Indian River	Blue Cypress	08-30-76	29.0	2	+	100	-
Levy	Stafford	08-30-76	30.0	2	-	100	-
	Richard	10-03-76	-	0.1	-	100	-
		07-09-77		3.2	+	300	-
Marion	Rousseau	08-30-76	28.0	2	-	100	-
	Half Moon	09-30-76	30.0	2	+	100	+
	George	09-30-76	26.0	2	-	100	-
Okeechobee	Okeechobee	09-09-76	32.0	189.2	+		
Pasco	Bell	08-30-76	32.0	2	+	100	+
Polk	Rudy	08-30-76	31.0	1	-	100	-
	Buffram	08-30-76	32.0	2	-	100	-
	Parker	02-10-76	19.0			100	-
		10-11-76	24.0	2	-		
Putnam	Oklawaha	09-30-76	28.0	2	-	100	+

(continued)

TABLE A-7 (continued)

County	Lake	Date	Water temp. °C	Samples: Water Size (L)	Samples: Water Results	Samples: Sediment Size (ml)	Samples: Sediment Results
Putnam	Crescent	09-30-76	27.0	2	-	100	+
Escambia	Escambia River	06-28-77	29.0	2	-	600	-
		08-16-77	27.0	2	-	400	-
Santa Rosa	Black Water Bayou	06-28-77	27.5	2	-	600	-
	Black Water River	08-15-77	30.0	2	-	400	-
Okaloosa	Yellow River	06-28-77	27.5	1	-	300	-
		08-16-77	26.0	1	-	200	-
	Sykes	06-28-77	32.5	1	-	300	+
Walton	Juniper	06-28-77	33.0	2	-	600	-
		08-16-77	29.5	2	-	400	-
Holmes	Cassidy	06-28-77	33.0	2	-	600	-
		08-16-77	31.0	2	-	400	-
Jackson	Marriett	06-28-77	31.0	1	-	300	-
		08-16-77	30.0	1	-	200	-
Washington	Blue	06-28-77	30.0	1	-	300	-
		08-17-77	31.5	1	-	200	-
	Sunnyhill	06-28-77	31.5	1	-	300	-
		08-17-77	32.0	1	-	200	+
Bay	White Western	06-28-77	31.0	1	-	300	-
		08-17-77	32.5	1	-	200	-
	Merial	06-28-77	31.0	1	-	300	-
		08-17-77	32.0	1	-	200	-
Gulf	Alice	06-28-77	32.5	1	+	300	-
	Dead	06-29-77	31.0	1	-	300	-
		08-17-77	32.0	1	-	200	-
Calhoun	Dead	06-29-77	31.5	1	-	300	-
		08-17-77	32.0	1	-	200	-
Liberty	Apalachacola River	06-29-77	31.0	2	-	600	-
		08-17-77	29.5	2	-	400	-
Leon	Bradford	09-13-76	28.5	189.2	-	200	-
		06-29-77	33.5	1	-	300	-
		08-18-77	30.5	1	-	200	-
	Ella	09-13-76	28.0	189.2	-	200	-
		06-29-77	36.0	1	-	300	-
		08-18-77	32.0	1	-	200	-

(continued)

TABLE A-7 (continued)

County	Lake	Date	Water temp. °C	Samples: Water Size (L)	Water Results	Sediment Size (ml)	Sediment Results
Jefferson	Miccosukee	06-29-77	32.0	1	-	300	-
		08-18-77	31.5	1	-	200	-
Taylor	Andrews	06-30-77	31.0	2	-	600	-
		08-18-77	32.0	2	-	400	-
Madison	Francis	06-30-77	32.0	2	-	600	-
		08-18-77	35.0	2	-	400	-
Hamilton	Suwanee River	06-30-77	25.5	2	-	600	-
		08-18-77	27.0	2	-	400	-
Suwanee	Suwanee	06-30-77	32.0	1	+	300	+
				1	-	300	-
Columbia	Alligator	06-30-77	37.0	1	-	300	-
		08-18-77	36.0	1	-	200	-
Baker	Ocean Pond	06-30-77	33.0	1	+	300	-
Union	Palestine	06-30-77	35.0	2	-	600	-
		08-18-77	28.5	2	-	400	-
Clay	Kingsley	06-30-77	31.0	1	-	300	-
		08-18-77	31.0	1	-	200	-
	Geneva	06-30-77	30.5	1	+	300	-
Alachua	Lockloosa	06-30-77	30.5	2	-	600	-
		08-18-77	32.0	2	-	400	-
	Newnans	08-27-77	29.0	1	-	200	-
Seminole	Brantley	10-05-77	29.5	189.2	+	200	+
	Bear	10-05-77	N.T.*	189.2	-	200	-

* Not taken

TABLE A-8. RESULTS OF ISOLATION ATTEMPTS FOR PATHOGENIC NAEGLERIA FROM 200 ML SEDIMENT SAMPLES FROM POTENTIAL CONTROL LAKES
(January, 1976 - March, 1978)

Lake	Date	Water temp.°C		Sediment sample	
		Bottom	Surface	Depth (meters)	No. positive/ no. tested
Howell	10-31-77		22.5	1.9	0/3
			22.5	3.0	1/3
			22.5	4.9	0/3
			22.5	5.2	0/3
			22.5	2.7	0/3
Little Pickett	10-20-77		22.5	6.4	0/4
			22.5	5.2	0/4
			22.5	7.3	0/4
			22.5	2.2	0/4
			22.5	6.1	0/4
			22.5	6.4	0/4
			22.5	5.5	1/4
			22.5	2.7	0/4
Big Pickett	10-06-77		28.0	0.3	0/1
	10-17-77		24.0	0.3	0/3
	10-20-77		22.5	7.0	0/2
			22.5	7.3	0/4
			22.5	4.0	1/4
			22.5	6.1	0/2
			22.5	7.6	0/2
Corner	10-06-77		27.0	0.3	0/1
	10-17-77		22.0	0.3	0/3
	10-24-77		22.0	4.9	0/3
			22.0	4.0	0/3
			22.0	3.3	0/3
			22.0	4.3	0/3
			22.0	5.5	0/3
	11-17-77		19.1	4.9	0/3
			19.1	4.3	0/3
			19.1	4.0	0/3
			19.1	3.3	0/3
			19.1	5.8	0/3
			19.1	4.6	0/3
	12-01-77		22.5	4.9	0/3
			22.5	3.3	0/6
			22.5	4.0	0/3
	12-01-77		22.5	4.3	0/3
			22.5	5.5	0/3

(continued)

TABLE A-8 (continued)

Lake	Date	Water temp.°C		Sediment sample	
		Bottom	Surface	Depth (meters)	No. positive/ no. tested
Corner (continued)	12-14-77		19.0	1.8	0/3
			19.0	2.7	0/6
			19.0	5.2	0/3
			19.0	5.5	0/6
	01-16-78	14.0	13.1	4.9	0/3
		16.2	15.0	5.2	0/6
		14.0	14.0	6.7	0/6
	04-06-78	17.5	25.5	4.6	0/3
				6.4	0/3
				4.6	0/3
				6.4	0/3
Jessup	10-05-77		27.0	<0.3*	0/1
	12-05-77		20.5	1.8	0/16
			20.5	1.5	0/4
	03-30-78		21.8	1.8	0/6
			22.5	1.8	0/6
			21.8	1.5	0/6
Little Lake Mary	10-05-77		27.0	<0.3*	0/1
	11-07-77		23.5	2.1	1/4
			22.5	4.0	6/12
			22.5	1.1	0/4
Beauclaire	11-28-77	21.0	18.5	3.3	0/4
		21.0	19.0	3.3	0/4
		21.0	19.0	3.6	0/4
		18.5	19.0	3.3	0/4
		18.5	19.0	2.4	0/4
	12-12-77	22.0	15.0	3.0	0/4
		21.0	15.0	3.0	0/4
		21.5	15.0	3.6	0/4
		15.0	15.0	2.7	0/4
		15.0	15.0	1.5	0/4
	01-09-78	16.0	15.5	3.0	0/18
	02-06-78	16.5		3.3	0/3
		15.8		3.3	0/3
		16.0		3.4	0/3
		12.0		3.3	0/3
		12.0		2.7	0/3
		12.5		3.0	0/3
		16.5		3.3	0/3
		15.8		3.3	0/3

(continued)

TABLE A-8 (continued

Lake	Date	Water temp. °C Bottom	Surface	Sediment sample Depth (meters)	No. positive/ no. tested
Beauclaire (continued)	02-06-78	16.0		3.4	0/3
		12.0		3.3	0/3
		12.0		2.7	0/3
		16.0		3.3	0/3
	02-23-78	16.0		3.6	0/3
		16.5		4.0	1/3
		11.8		3.0	0/3
	03-16-78	19.8		4.0	0/9
Lawne	12-19-77	19.0	19.0	3.6	0/3
		19.0	19.0	2.1	0/3
		18.5	19.0	4.9	0/3
		20.0	19.0	2.4	0/3
		17.0	19.0	6.1	0/3
		18.0	19.0	4.9	0/3
	03-27-78	18.0		4.9	1/8
		17.0		7.3	1/4
East Lake Tohopekaliga	01-12-78	12.0	12.0	3.0	0/3
		13.0	13.0	3.0	0/3
		13.8	13.0	4.3	0/3
		13.0	13.0	3.3	0/3
		13.0	13.2	3.0	0/3
		13.8	13.2	4.0	0/3
Harris	01-19-78	14.0	13.0	5.8	1/12

* 189.2 liters of water tested on same date were negative.

TABLE A-9. SEDIMENT SPECIMEN POSITIVITY BASED ON WATER DEPTH AT SAMPLING SITE
(January, 1976 - March, 1978)

Depth (meter)	January		February		March		April		May		June	
	1	2	1	2	1	2	1	2	1	2	1	2
0-1	0/1	0	0/6	0	0/14	0	0/17	0	0/15	0	5/29	17.2
1.1-2	0/7	0	0/3	0	0/18	0	0/1	0	NS	--	NS	--
2.1-3	2/42	4.8	2/61	3.3	3/28	10.7	1/12	8.3	0/30	0	2/16	12.5
3.1-4	0/39	0	1/45	2.2	NS	---	0/13	0	NS	--	0/4	0
4.1-5	0/6	0	NS	--	1/8	12.5	0/6	0	1/4	25.0	1/4	25.0
5.1-6	16/45	35.5	8/14	57.1	6/16	37.5	3/9	33.3	3/8	37.5	NS	--
6.1-7	0/9	0	15/15	100.0	18/22	81.8	4/12	33.3	4/4	100.0	5/8	62.5
7.1-8	10/10	100.0	5/9	55.5	5/8	62.5	2/3	66.7	4/4	100.0	NS	--
8.1-9	0/3	0	6/9	66.7	5/6	83.3	NS	--	3/4	75.0	0/4	0
9.1-10	1/4	25.0	NS	--	1/4	25.0	NS	--	NS	--	NS	--

	July		August		September		October		November		December	
	1	2	1	2	1	2	1	2	1	2	1	2
0-1	5/25	20.0	7/12	58.3	4/17	23.5	2/28	7.1	0/21	0	NS	--
1.1-2	NS	--	NS	--	NS	--	0/3	0	0/10	0	0/29	0
2.1-3	13/13	100.0	NS	--	NS	--	1/14	7.1	6/24	25.0	10/74	13.5
3.1-4	NS	--	NS	--	NS	--	0/3	0	0/19	0	0/23	0
4.1-5	NS	--	NS	--	NS	--	0/9	0	6/32	18.7	0/16	0
5.1-6	NS	--	NS	--	NS	--	0/14	0	3/11	27.3	0/12	0
6.1-7	NS	--	NS	--	NS	--	0/19	0	2/4	50.0	5/9	55.5
7.1-8	NS	--	NS	--	NS	--	1/16	6.2	NS	--	2/2	100.0
8.1-9	NS	--	NS	--	NS	--	NS	--	NS	--	NS	--
9.1-10	NS	--	NS	--	NS	--	NS	--	NS	--	0/4	0

Totals Depth	0.1	1.1-2	2.1-3	3.1-4	4.1-5	5.1-6	6.1-7	7.1-8	8.1-9	9.1-10
Col. 1	23/185	0/71	40/314	1/146	9/85	39/129	53/102	29/52	14/26	2/12
Col. 2	12.4	0	12.7	0.7	10.6	30.2	52.0	55.8	53.8	16.7

Column 1 - Number specimens positive/number tested.
Column 2 - Percent positive.
NS - No specimen taken.

TABLE A-10. CHEMICAL DATA - POSITIVE LAKES* IN GEORGIA

Date 1978	Lake	Water temp. °C	D.O. mg/L	Hard $CaCO_3$ mg/L	Hard Ca mg/L	pH	Acid. free $CaCO_3$ mg/L	Acid. total $CaCO_3$ mg/L	Alk. phen. $CaCO_3$ mg/L	Alk. total $CaCO_3$ mg/L	Cl^- mg/L	NO_3 mg/L	NO_2 mg/L	PO_4 ortho mg/L	PO_4 organ. mg/L	SO_4 mg/L
8-7	Griffin	29.0	6.3	10.0	5.0	5.5	0	30.0	0	5.0	10.0	0.2	<0.001	0.06	0.65	2.5
8-7	Cypress	29.0	7.6	5.0	5.0	5.3	0	30.0	0	5.0	10.0	<0.1	<0.001	0.04	0.60	<0.1
8-8	Carrol	30.0	6.8	10.0	10.0	5.5	0	15.0	0	20.0	5.0	<0.1	<0.001	0.10	0.90	
8-8	Olmstead	32.0	5.6	15.0	15.0	5.5	0	11.0	0	25.0	5.0	<0.1	<0.001	0.10	4.20	2.0
8-13	Spivey	27.0	7.4	15.0	10.0	5.6	0	20.0	0	15.0	5.0	<0.1	<0.001	0.04	1.22	4.0
8-14	Robin	28.0	7.8	10.0	10.0	5.4	0	15.0	0	10.0	5.0	<0.1	<0.001	<0.01	1.4	<0.1
Average		29.2	6.9	10.8	9.2	5.5	0	20.2	0	13.3	6.7	<0.1	<0.001	0.06	1.5	1.7

* Specimen from Long Pond broken in transit.

TABLE A-11. CHEMICAL DATA - NEGATIVE LAKES IN GEORGIA

Date 1978	Lake	Water temp. °C	D.O. mg/L	Hard $CaCO_3$ mg/L	Hard Ca mg/L	pH	Acid. free $CaCO_3$ mg/L	Acid. total $CaCO_3$ mg/L	Alk. phen. $CaCO_3$ mg/L	Alk. total $CaCO_3$ mg/L	Cl^- mg/L	NO_3 mg/L	NO_2 mg/L	PO_4 ortho mg/L	PO_4 organ. mg/L	SO_4 mg/L
8-9	Deltina	31.0	10.6	5.0	5.0	5.7	0	30.0	0	15.0	5.0	0.2	<0.001	0.01	0.4	<0.1
8-9	Lester	28.5	8.5	7.5	5.0	5.5	0	20.0	0	15.0	5.0	0.3	<0.001	0.12	0.75	<0.1
8-10	Fort Yargo	27.0	7.0	10.0	10.0	5.5	0	20.0	0	20.0	5.0	<0.1	<0.001	<0.01	0.9	20.0
8-10	Yonah	25.0	6.8	5.0	5.0	5.7	0	20.0	0	15.0	2.5	<0.1	<0.001	0.1	0.44	<0.1
8-11	Sky	24.0	6.7	5.0	5.0	5.3	0	25.0	0	10.0	5.0	<0.1	<0.001	0.25	0.6	<0.1
8-11	Dockery	21.0	5.3	5.0	5.0	5.5	0	30.0	0	10.0	5.0	0.1	<0.001	0.08	0.65	<0.1
8-12	Blackburn	24.5	7.2	5.0	5.0	5.7	0	15.0	0	10.0	2.5	<0.1	<0.001	0.05	0.69	<0.1
8-12	Indian	30.5	7.5	10.0	10.0	5.3	0	10.0	0	15.0	2.5	<0.1	<0.001	0.05	4.0	2.0
8-13	Ransby	33.0	8.8	10.0	5.0	5.5	0	25.0	0	15.0	2.5	<0.1	<0.001	0.05	0.7	2.0
8-14	Concharty	29.0	7.4	0	0	5.3	0	15.0	0	5.0	2.5	<0.1	<0.001	0.02	1.3	
8-15	Tobesofkee	29.0	7.9	15.0	10.0	5.7	0	15.0	0	15.0	5.0	<0.1	<0.001	0.05	0.55	<0.1
8-15	Houston	33.5	8.5	5.0	5.0	5.7	0	30.0	0	10.0	7.0	<0.1	<0.001	0.06	4.2	<0.1
8-16	Blackshear	31.0	8.8	15.0	10.0	5.7	0	15.0	0	15.0	5.0	<0.1	<0.001	0.02	1.16	2.0
Average		28.2	7.8	7.5	6.1	5.5	0	20.8	0	13.1	4.2	<0.1	<0.001	0.06	1.26	2.2

TABLE A-12. CHARACTERISTICS OF SEROPOSITIVE NONPATHOGENIC AND PATHOGENIC *NAEGLERIA*

Characteristics	Conditions when observed	Seropositive nonpathogen	Pathogen
Plaque	At 37°C and 43°C on inorganic agar seeded with *E. aerogenes*	Large Dispersed Diffuse plaque line	Small Aggregated Sharp plaque line
Antigens	Common and specific antibodies as shown by fluorescent antibody tests	Titers	Titers
	Homologous antiserum	320*	640
	Heterologous antiserum	320	160
	Cross absorbed homologous antiserum	40	160
	Cross absorbed heterologous antiserum	0	0
Culture	Growth in Chang's medium	No growth - survival only	Logarithmic
	Growth in Chang's medium with bacterial homogenate added	Logarithmic	Logarithmic
	Trophozoites under anaerobiasis	Death	Death
	Cysts under anaerobiasis	Survival	Survival
Mouse pathogenicity	Intranasal instillation	No deaths	Deaths in 5 days
	Intracerebrally	No deaths	Deaths in 4 days
Cyst wall	Sonication at 100 watts for 120 seconds	46.5% intact	27.3% intact
	Electronmicroscopy	Thick	Thin (?)
	Phase contrast microscopy	Pores	No pores

* Reciprocal of serum dilution

APPENDIX B

CHANG'S MEDIUM - CALF SERUM-YEAST-EXTRACT-CASEIN MEDIUM (CSYECM) FOR AXENIC CULTIVATION OF PATHOGENIC NAEGLERIA

#1	Per 500 ml	1000 ml
Isoelectric Casein	5.0 g	10.0 g
$Na_2HPO_4 \cdot 7\ H_2O$	1.25 g	2.5 g
(If Na_2HPO_4 anhydrous)	(0.6625)	(1.325)
Dissolve in of distilled water using a 2000 ml flask	400 ml	800 ml
#2		
Dextrose	1.25 g	2.5 g
KH_2PO_4	0.4 g	0.8 g
Dissolve in of distilled water using a 250 ml flask	40 ml	80 ml

Autoclave #1 and #2 for 15 minutes and allow to cool.

#3		
Yeast extract	2.5 g	5.0 g
Dissolve in of distilled water	10 ml	20 ml

Note: Due to loss in filtration procedure for sterilization, use 10 g yeast and 40 ml distilled water. Use 20 ml of the filtrate for preparation of medium.

#4		
Fetal Calf serum	50 ml	100 ml
#5		
Penicillin (stock) 200,000 U/ml	0.5 ml	1.0 ml
Streptomycin (stock) 200,000 µg/ml	0.5 ml	1.0 ml

Using aseptic technique, pour the yeast extract preparation into the dextrose, add the antibiotics and swirl. Pour this mixture into the casein and add fetal calf serum. Mix well. Distribute aseptically, 5 to 10 ml amounts into 16 X 125 mm sterile screw-capped tubes. Incubate for 2 days at 37°C to check sterility.